PARASITOLOGY

(*Founded by G. H. F. Nuttall, F.R.S.*)

EDITED BY

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IN CONJUNCTION WITH

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OPISTHOLEBES DIODONTIS N.SP., ITS DEVELOPMENT IN THE FINAL HOST, THE AFFINITIES OF SOME AMPHISTOMATOUS TREMATODES FROM MARINE FISHES AND THE ALLOCREADIOID PROBLEM

BY R. M. CABLE*

Department of Biological Sciences, Purdue University, Lafayette, Indiana, U.S.A.

(With 8 Figures in the Text)

INTRODUCTION

In examining marine fishes of Puerto Rico for parasites, the porcupine fish, *Diodon hystrix*, was almost always found to harbour a species of *Opistholebes*, sometimes in considerable numbers. Two fish yielded a series from still encysted metacercariae in the stomach contents to large adults in the intestine. A study of this material has shown it to represent a new species and has yielded significant information concerning not only the affinities of the Opistholebetidae, but also the interpretation of the position of the ventral sucker in that family. The genus *Maculifer* is a typical distome with a well-developed hind-body, whereas in *Opistholebes* the ventral sucker is at the posterior end of the body; *Heterolebes* is intermediate in that respect. A parallel situation exists in two other families of trematodes parasitizing marine fishes, viz. the Gyliauchenidae and the Cephaloporidae. As in the Opistholebetidae, those families contain genera showing gradations between the distome and amphistome body plan. Ozaki (1937) made an excellent study of the families and characterized them and their genera clearly for the first time. Although his observations on the Gyliauchenidae are especially enlightening, exception is taken to his views concerning the superfamily status of that group and the amphistome body plan in phylogeny as will be brought out in later discussion.

In this study, much use was made of living material, especially in observing the excretory system. For other morphological details, stained whole mounts and serial sections were prepared. The scale drawing of the adult was made by microprojection, and all measurements are in millimetres. Other species needed for comparison with the present one were kindly provided by Prof. H. W. Manter and Dr E. W. Price.

OBSERVATIONS

Opistholebes diodontis n.sp.

ADULT (Figs. 1, 2, 7 and 8)

DIAGNOSIS. With the characters of the genus *Opistholebes* Nicoll, 1915. Body oval, thick, somewhat flattened and very muscular. Cuticle thick and wrinkled

* Fellow, John Simon Guggenheim Memorial Foundation, 1951–2. This study was made possible by facilities generously provided by the Department of Biology at the College of Agriculture and Mechanic Arts, University of Puerto Rico.

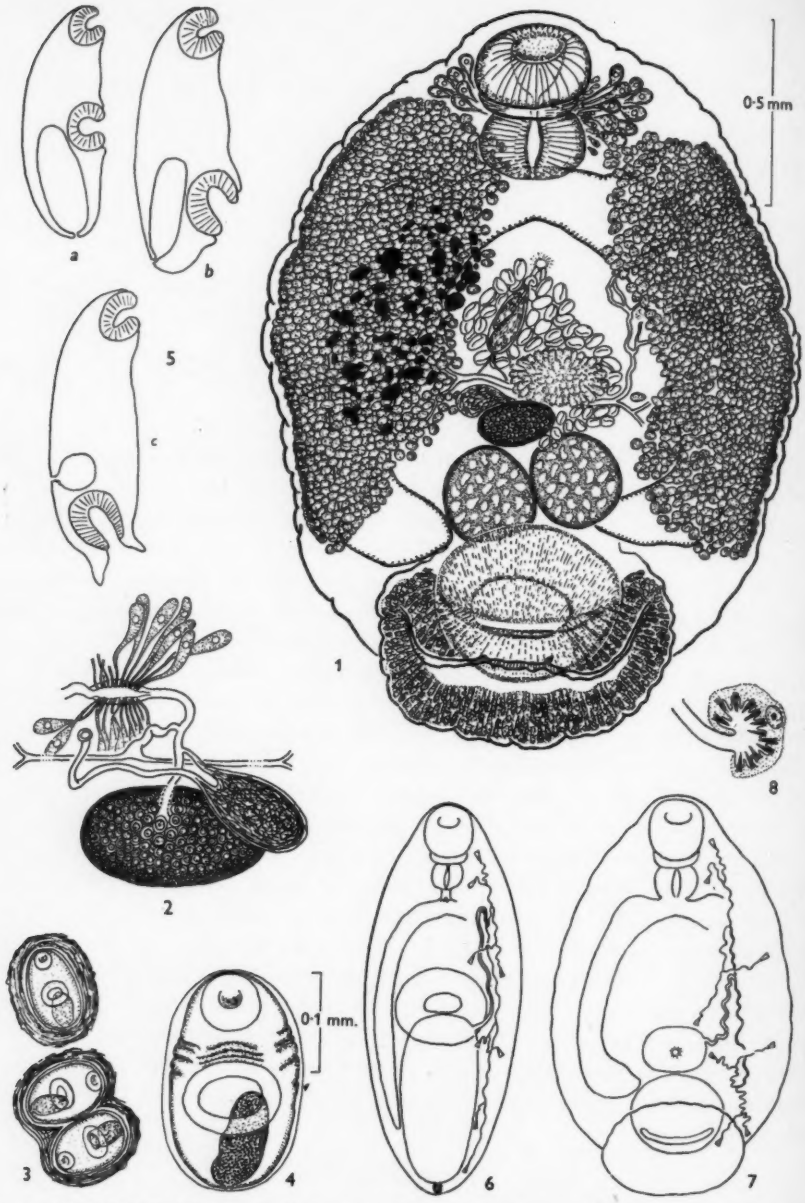

Fig. 1. Adult *Opistholebes diodontis* in ventral view. Specimen of average size with pigment spots developed only on right side. In old specimens, pigment obscures vitellaria.
Fig. 2. Female complex in dorsal aspect drawn freehand from a living specimen.
Fig. 3. Metacercariae from stomach of host.
Fig. 4. Metacercaria with fibrous outer cyst removed.
Fig. 5. Diagrams showing progressive shifting of ventral sucker and excretory vesicle as the young worm grows.
Fig. 6. Excretory system of metacercaria.
Fig. 7. Excretory system of adult.
Fig. 8. Complex flame cell of large, pigmented adult.

but without spines. Posterior disk prominent and very glandular with heavy muscles extending anteriorly from it into the body. Smallest mature specimen 1·48 long and 1·30 wide, largest 2·52 by 1·95. Oral sucker wider than long, measuring 0·16–0·31 by 0·28–0·45. Ventral sucker close to posterior end of worm and somewhat wider than long, attaining a maximum transverse diameter of 0·63 and a length of 0·54; aperture wide, slit-like, directed postero-ventrally and opening into a depression within the surrounding disk. In flattened specimens, anterior half of disk almost always folded over and superimposed on posterior half; sometimes containing masses of host tissue, thus suggesting a haptoral function for disk as well as sucker. Pharynx 0·14–0·24 long and 0·21–0·34 wide, close to muscular post-oral ring of oral sucker, a pre-pharynx being evident only in sections. Oral sucker and pharynx flanked by several large and numerous small unicellular glands which stain deeply and obscure post-oral ring. Oesophagus absent, cæca wide, arching laterally from pharynx, extending posteriorly and then medially to terminate close to testes; cæca with a distinct cellular lining and prominent outer muscle layer. Testes smooth, spherical to oval, and 0·22–0·34 in diameter; symmetrical or slightly diagonal in placement, immediately anterior to ventral sucker or slightly overlapping that organ dorsally. Cirrus sac slightly to right of mid-line and well anterior to ovary; with elongate oval posterior portion and long, tubular and often sinuous ejaculatory region not extending anterior to genital pore. Seminal vesicle tubular and coiled, prostate poorly developed. Genital pore median or slightly to left, from intestinal bifurcation to a point well posterior to that level. Ovary transversely elongate, from 0·094–0·17 to 0·17–0·29; situated immediately anterior to testes, either in mid-line or distinctly to right but never directly anterior to right testis. Oviduct extends anteriorly from ovary and joined on right by Laurer's canal enlarged to form a prominent seminal receptacle dorso-lateral and anterior to ovary. After receiving vitelline duct, oviduct turns to left to join thick-walled ootype. Mehlis's gland extremely prominent, larger than ovary, and mostly dorsal to ootype. Uterus largely to left of mid-line, between testes and intestinal bifurcation, crossing to right anteriorly; metraterm arches postero-ventrally to open at genital pore through shallow atrium. Eggs thin-shelled, 0·063–0·071 by 0·047–0·050 in living specimens; collapsed and a little wider (0·055) in totomounts. Vitellaria extensive with follicles in a dense, uninterrupted zone on each side from pharyngeal level to or beyond posterior terminations of cæca; zones well separated medially, their follicles not overlapping gonads; vitelline ducts prominent. Pigment spots variable, but number generally correlated with size of worm and almost obscuring vitellaria in large specimens; spots from white to almost black in reflected light and not altered in appearance by reagents used in preparing totomounts and sections. Excretory vesicle and pore dorsal to triangle formed by ovary and testes. Vesicle transversely elongate in mature specimens, each side receiving a main excretory tubule which extends anteriorly a short distance and receives an anterior and a posterior collecting tubule. Each collecting tubule is joined by two groups of flame cells, two per group, so that the excretory formula is 2 $((2+2)+(2+2))$. Flame cells of usual appearance in young specimens but becoming complex with many cones of cilia (Fig. 8) as pigment spots appear.

HOST. *Diodon hystrix.*

SITE. Posterior intestine.

LOCALITY. Coastal waters of Mona Passage, Puerto Rico.

TYPE SPECIMENS. Holotype and paratype No. 37440. Helminthological Collection, U.S. National Museum.

A comparison of *Opistholebes diodontis* with other species of the genus shows a number of differences. To stress all of them would require an extensive table or lengthy discussion. For that reason, the Key given by Ozaki (1937) is emended to distinguish the species.

Key to species of Opistholebes

1. Well developed glandular disk present 3
   Glandular disk absent 2
2. Body oval, ovary anterior to right testis, cirrus sac 0·45 mm. long
   O. amplicoelus Nicoll, 1915
   Body more elongate, ovary submedian toward right testis, cirrus sac 0·65 mm. long
   O. elongatus Ozaki, 1937
3. Posterior end of cirrus sac close to ovarian level, vitelline follicles meeting anterior to genital pore or overlapping gonads posteriorly 4
   Cirrus sac well removed from ovary, vitelline zones neither coalescing anteriorly nor overlapping gonads posteriorly **O. diodontis** n.sp.
4. Body up to 3·8 mm. long, ovary submedian, vitelline follicles coalesce anteriorly, ejaculatory duct almost straight **O. cotylophorus** Ozaki, 1935
   Body up to 1·35 mm. long, ovary anterior to right testis, vitelline follicles do not meet anteriorly but overlap gonads posteriorly, ejaculatory duct strongly recurved.
   O. adcotylophorus Manter, 1947

Probably as significant as morphological dissimilarities are differences in host species and localities that are widely separated with deep water between. These conditions would be strong factors of isolation and hence expected to favour speciation in this genus of trematodes, restricted as it is to a single host group of shallow-water fish. Geographically, the species nearest *Opistholebes diodontis* is *O. adcotylophorus* found at Tortugas, Florida. While the distance between that locality and Mona Passage is not great, the deepest water of the Atlantic lies between and would seem to serve as an effective barrier to the intermingling of shallow-water hosts and their parasites.

As Manter (1947) found but two specimens of *O. adcotylophorus*, the fact that they were much smaller than the size attained by *O. diodontis* is of no significance *per se* for these worms obviously become mature well before reaching their maximum size. However, in comparing individuals of the two species having the same body size, it is found that *O. adcotylophorus* is fully mature with many eggs in the uterus and body pigment present, whereas *O. diodontis* is still functionally immature and lacks pigment. Other respects in which these species differ include the position of the ovary, median extent of vitelline follicles, course of the ejaculatory duct, location and prominence of Mehlis's gland, shape of the oral sucker and species of host.

METACERCARIA AND ITS DEVELOPMENT TO THE ADULT

Numerous metacercariae (Figs. 3, 4) were recovered from the stomachs of two fish. The worms occurred either singly or in groups of two or more within a common, thick-walled fibrous cyst evidently of host origin. Immediately surrounding each

worm was a thin cyst, oval in outline, and measuring 0·22 by 0·15. The fluke itself was a typical distome. The cuticle was unarmed but thick and wrinkled, there was no indication of eye-spot pigment, and little development of the reproductive system had occurred. The excretory pore was posterior in position; the vesicle was large, sac-shaped, and filled with opaque granules. It almost filled the hindbody and overlapped the posterior edge of the ventral sucker. The flame cell formula was the same as described for the adult.

The metacercariae differed in no respect from that stage of several opecoelids the writer has studied. There is every indication short of controlled feeding experiments, however, that the encysted worms were to develop into adults of *O. diodontis*. In the stomach and upper intestine free distomes, identical to those removed from cysts, were found, and along the intestine all sizes up to large, heavily pigmented adults were present. Stomach contents showed that the fish had been feeding largely on sea-urchins. Several urchins taken later where one of the fish was speared were examined without finding metacercariae, but the number was too small to be indicative one way or the other.

With growth (Fig. 5), the worm changes from the distome form of the metacercaria to the adult with the ventral sucker at the posterior end of the body. This change is interpreted as failure of the hindbody to grow while the remainder of the worm increases almost twenty times in length. As a result, organs which occupy the hindbody of distomes lie in what corresponds to the forebody of such forms. Late development of the reproductive system probably facilitates this topographical arrangement. The cæca which at first extend well into the hindbody and taper at their ends (Fig. 6), shorten, and become wider as they retreat to the preacetabular region. The situation here is very different from that of the gyliauchenids in which there may be a compensatory elongation and coiling of the prepharynx as the cæca shorten in proportion to the extent to which the ventral sucker is shifted toward the posterior end of the body. In *Opistholebes*, the excretory vesicle moves anteriorly, and with the disappearance of the hindbody, the excretory pore shifts until it is dorsal.

That such a transformation might occur is suggested by the position of the ventral sucker in other genera of the Opistholebetidae. The present study demonstrates the direction in which the transformation moves in ontogeny and shows that the situation is quite different from that of certain paramphistomes in which the ventral sucker of the cercaria is at the posterior end of the body and remains there during development to the adult.

DISCUSSION

Until the life history of one or more opistholebetids is known, the affinities of the group cannot be established beyond question. However, the present study gives the strongest evidence short of such information and points very definitely to a close affinity with the Opecoelidae. *Maculifer* differs from an opecoelid only in having a post-oral ring and pigment spots; the reproductive system and the topography of its parts are the same. Nicoll (1915) and Price (1937) recognized this resemblance; because of it Cable & Hunninen (1942) expressed the possibility

that the Opistholebetidae may be related to the Opecoelidae. The present study shows that agreement extends as well to the excretory system, a recognized basis for determining relationships. It further demonstrates that the posterior location of the ventral sucker in *Opistholebes* is secondary in ontogeny and probably also in phylogeny of the group.

With the information at hand, it may be predicted that the cercaria of *O. diodontis*, when found, will be a distome lacking eye-spots and body spines but having a thick, probably warty or wrinkled cuticle. The excretory vesicle will be broadly sac-shaped and confined to the hindbody. The pattern of the excretory tubules is known and the flame cell formula will be $2\,[(n+n)+(n+n)]$ with not more than two flame cells per group (n). Characteristics of the digestive system, if developed, are known. In short, one need only to add the tail and larval glands and know the nature of the bladder wall and whether a stylet is present to complete a general description of the cercaria.

Marine cercariae are far less perfectly known than fresh-water larvae and there still may be unrecognized larval types. It is very doubtful, however, that further studies will reveal enough such types to account for the various proposed families of trematodes and not demonstrate a very close relationship of most if not indeed all of those families for whom life histories are still unknown to groups for which at least some information in that respect is at hand. It therefore is significant that of the recognized cercariae, only the cotylomicrocercous larvae, many of which are known to be cercariae of the Opecoelidae, can qualify in all features as larvae of the Opistholebetidae as well. This fact and the agreement between the adults of these families in so many respects leaves no doubt that they are more closely related to each other than either is to any other family of trematodes.

The pigment spots peculiar to the Opistholebetidae have been interpreted as a lymph system to bring that family into line with the paramphistomes and gyliauchenids which possess such a system. However, Ozaki (1937) concluded from histochemical studies that the spots are cells containing accumulated metabolic by-products of purine nature which the worms are not otherwise able to handle. The writer fully agrees with that interpretation and ventures to suggest that the structures supplement excretory function because these thick-bodied worms are especially poor in flame cells when compared with other trematodes of like mass. An unusual demand on excretory function is indicated by the fact that as the worms become large in size and pigment spots develop, the flame cells become enormous and acquire many cones of cilia (Fig. 8). In sections of large worms, granules of pigment are scattered throughout the parenchyma, whereas such granules are lacking in younger specimens. All pigmented opistholebetids occur in puffers, and it may be that those hosts impose on the parasites metabolic demands not encountered by related trematodes in other fishes.

After complex flame cells were found in *O. diodontis*, and because of its evident relationship to opecoelids, such flame cells were sought in thick-bodied opecoelids and were quickly found in a species identified at the time as *Plagioporus crassigulus* (Linton) from a porgy (*Calamus* sp.). Later, a study of whole mounts and sections showed that the species possessed a post-oral ring and other features, whereby

it differed from the opistholebetid genus, *Maculifer*, only in lacking pigment spots. It was then suspected that the same might be true of *Plagioporus gastrocotylus* Manter, 1940, also from a porgy. Materials of that species kindly loaned by Prof. Manter not only showed a post-oral ring, but also revealed that the fleshy disk surrounding the ventral sucker of that species is undoubtedly homologous to the glandular disk characterizing certain species of *Opistholebes* and especially well developed in *O. diodontis*.

Manter (1947) was hesitant in leaving *Plagioporus crassigulus* in the genus *Plagioporus*, and later (1954) erected for that species and *P. gastrocotylus* the genus *Pachycreadium*, which he assigned to the Opecoelidae. It is now evident that these forms belong to the Opistholebetidae, and it is doubtful whether *Pachycreadium* is distinct from *Maculifer*, pigmentation being the only morphological distinction. However, there is some evidence that pigment is stored in specialized cells rather than by parenchymal cells in general, and it may be that such specialized cells are absent in the species assigned to *Pachycreadium*. Because of that possibility and differences in definitive hosts (porgies for *Pachycreadium* and puffers for *Maculifer*) the genus *Pachycreadium* is retained for the present, although further study may require that it be reduced to synonomy with *Maculifer*.

Ozaki (1937) fully appreciated the polyphyletic character of amphistomatous trematodes, and although he assigned the Gyliauchenidae to the Paramphistomoidea, he set the Opistholebetidae and Cephaloporidae apart from that superfamily. Because of the tendency of many of these parasites to localize in the posterior end of the intestine and associated organs, Ozaki interpreted 'this locality of inhabitation...as the first step to entoparasitism'. He further stated that the various types of amphistomes 'started independently and took each its own evolutional course to distomes'. In the writer's opinion, these views are wholly untenable. All evidence indicates that the amphistomatous condition is secondary in the phylogeny of the Opistholebetidae and probably also the Gyliauchenidae and Cephaloporidae as well. Even among the Paramphistomoidea there are genera in which the ventral sucker is considerably removed from the posterior end of the body. In that group, it may well be that the tendency of the sucker to shift posteriorly has been more uniform and pronounced than in others and has become impressed on the cercaria as well as the adult stage.

It probably was because the gyliauchenids possess lymph channels that Ozaki (1937) assigned that group to the Paramphistomoidea. As discussed by Cable & Hunninen (1942), there is good evidence, mostly from Ozaki's excellent paper, that the gyliauchenids are aberrant lepocreadioids, a complex that now seems to include also the Megasolenidae and Megaperidae. The megasolenids, like the gyliauchenids, possess lymph channels and so do the lepocreadiid genera *Apocreadium* and *Choanodera*.

In discussing the Cephaloporidae, Ozaki (1937) stated, '*Prosthogonismus* (sic) may be considered derived from *Plectognathotrema lobatum* by the advance of the acetabulum.' Although the reverse of that situation seems more probable, a close relationship between those forms may indeed exist. If such an affinity were proved, then the Cephaloporidae would belong to the Plagiorchioidea, a large superfamily

otherwise scarcely represented among the trematode parasites of marine fishes. Ozaki did not mention one interesting and possibly significant fact, viz. that, like *Prosthogonimus*, all known cephaloporids inhabit the reproductive system of their hosts which, however, are not birds but fishes of a single family, the file fishes. Price (1937) regarded *Cephaloporus* as an aberrant representative of the Zoogonidae, and *Plectognathotrema* as having a similar status with respect to the Fellodistomatidae; recent studies indicate that those families are not at all closely related. That the Cephaloporidae is an aberrant family derived from a single better known and more generalized group seems certain but the identification of that group must await more precise knowledge of adult morphology and information concerning the life history in the Cephaloporidae.

It is of interest to note that a posterior movement of the ventral sucker evidently occurs in still another group, the Hemiuridae, if its members become far displaced from the usual site in the definitive host. Most hemiurids of fishes localize in the upper digestive tract of their host, usually the oesophagus and stomach and have powerful suckers rather close together and a long hindbody; a few short-bodied genera occur in the intestine. Recently, however, Wolfgang & Myers (1954) have described a hemiurid from the ovaries of marine fishes, and although the tendency toward the amphistome condition in that form is less pronounced than in other groups discussed above, the ventral sucker is farther posterior and the hindbody correspondingly shorter than in hemiurids inhabiting the digestive tracts of their hosts.

Skrjabin (1949) was not consistent in his concept of the taxonomy of amphistomes. He recognized the affinities of the microscaphidiid monostomes to paramphistomes, as pointed out long ago by Odhner, but disregarded the many fundamental respects in which opistholebetids differ from paramphistomes and gave unwarranted significance to the position of the ventral sucker in elevating the opistholebetids to superfamily rank and including them in the order Paramphistomatata. A natural system of classification, one that expresses phylogeny, is scarcely served by either such an arrangement or the practice of erecting new families and superfamilies on the basis of adult characters which life-history studies have shown to be highly variable in closely related genera.

In considering taxonomic expression of the relationship between the Opistholebetidae and Opecoelidae, one is immediately faced with one of the major unsolved problems in the systematics of the digenetic trematodes, viz. affinities in the vast group that for lack of a better term may be called the allocreadioid trematodes. To assign the Opistholebetidae to that assemblage would add to the confusion and not signify that the family is more closely related to the Opecoelidae than to other allocreadioids.

It is evident from life-history studies that if this complex is a natural one, its cercariae have attained a diversity of structure and host relationship not approached by the larvae of any of the better understood groups. On the other hand, if the group is polyphyletic, as the writer believes, then the adults have in some instances evolved convergently to the extent that in gross structure it is not always easy to distinguish between genera that may actually represent distinct families or even

superfamilies. The present situation in these trematodes may well be comparable to that which existed in the Heterophyidae 30 years ago. Since that time, life-history studies and more precise knowledge of adult morphology have demonstrated that family to have been an utterly unnatural one to which had been assigned trematodes now known to belong to three superfamilies, two in the order Prosostomata and one in the Strigeatoidea!

Life-history studies indicate that there may be three major groups within the allocreadioid complex, the allocreadioids, the lepocreadioids, and the opecoeloids. Although Dollfus (1949) regards the first two as 'true' allocreadioids and the opecoelids as 'false' ones, it is the writer's belief that the complex will eventually prove to contain three superfamilies. The allocreadioids in the strict sense have ophthalmoxiphidiocercariae which bear simple tails, and develop in lamellibranchs; the adults are largely if not exclusively parasites of fresh- and brackish-water fishes. Known lepocreadioid cercariae are biocellate, lack a stylet, have variably ornate tails, and develop in prosobranch gastropods, some fresh-water but largely marine. Some of these larvae are trichocercous and resemble superficially certain cercariae of the Fellodistomatidae which develop in marine lamellibranchs. The opecoelid cercaria possesses a stylet, lacks eye-spots and body spines, has a short glandular tail, and develops in prosobranch snails. The adults are well represented in both fresh-water and marine fishes.

Dollfus (1913) expressed the view that the cotylomicrocercous larvae of the opecoelids are a true larval type and that the term *de moignon* implying that the tail is stumpy and poorly developed is incorrectly applied to these cercariae. It is common knowledge that in some larval types, the tail may be well developed in one species, reduced in another, or sometimes even lacking. Such variation has never been observed in opecoelid larvae, however, and knowledge of that group is considerable. The writer therefore has agreed with Dollfus and recognized the Opecoelidae as a distinct family, but not for the reason that was first proposed by Ozaki who considered anal openings as family characters. Some investigators, however, still regard the opecoelids as belonging to the Allocreadiidae.

In cercariae of the three groups, the excretory vesicle is usually sac-shaped and is described as being thick-walled, but the wall differs not only in thickness but in its character as well. In ophthalmoxiphidiocercariae of the Allocreadiidae, the bladder wall contains cuboid to columnar gland-like cells, the whole having an irregular or scalloped contour and a smooth-walled lumen devoid of concretions. In lepocreadiid cercariae, the bladder wall is much thinner, with indistinct finely granular block-like masses, evidently cells, or sometimes appearing to consist of little more than a membrane with a thin, adherent granular layer on the outside; as a rule the lumen contains refractile concretions. In opecoelid cercariae, the wall of the excretory vesicle is thick with regular outer contour, but often with a very uneven lumen due to protrusion of the large cells of the wall into it. Concretions are absent. Furthermore, the flame-cell pattern is simpler in this cercarial type than in the others, there being but four groups on each side of the body with, as a rule, two flame cells per group. This difference in complexity of the excretory system becomes even more pronounced in the adult stages. There is much need for a critical

study of the changes that occur in respect to the excretory vesicle in the metacercaria. After all, there are other types of cercariae that have thick-walled bladders, the larvae of the opisthorchioids, hemiurids, and gorgoderids for example, and undue significance may have been placed on that character in attempting to deal with the allocreadioid complex. While it is true that the embryonic excretory pores occur at the junction of the body and tail in the otherwise diverse larval types of that group, the pores are similarly located in certain other cercariae as well, e.g. the vast number of plagiorchioid larvae.

Because of the distinctive features of the morphology and host relationship of larval forms, and certain aspects of adult structure as well, the writer ventures to take a step toward resolving the 'allocreadioid problem' by proposing that a superfamily be erected to include the Opecoelidae and Opistholebetidae and thus separate them as closely related families from the remainder of the complex. The only alternative that would express such an affinity and at the same time set those groups apart from the others would be to broaden the definition of the Opecoelidae to include the Opistholebetidae as a subfamily. That arrangement does not seem advisable because it would place the opistholebetids on a footing equivalent to that of subfamilies that have been proposed for the Opecoelidae.

It is at present impossible to resolve the problem further because of the impasse created by the life cycles reported for *Allocreadium isoporum* and *A. ictaluri*. The first species is supposed to have an ophthalmoxiphidiocercaria developing in a lamellibranch (Looss, 1894) and encysting in the mollusk (Dollfus, 1949), but to the writer's knowledge that cycle has not been demonstrated experimentally; such a study urgently needs to be made. In investigating the life cycle of *A. ictaluri*, Seitner (1951) reported a cercaria of the lepocreadiid type developing in a gastropod and encysting in clams. It seems impossible that both accounts can be correct. Seitner's study was made under the writer's direction with what seemed to be adequate controls but the problem is being re-investigated to eliminate any possibility of error. The cercaria he reported for *A. ictaluri* is so similar in fundamental respects to the larva of *Deropristis inflata*, a lepocreadiid, that it could well be the cercaria of the closely related *Skrjabinopsolus manteri* (see Cable, 1955), a common parasite of the sturgeon in the waters from which infected snails used by Seitner were collected.

If the life cycles of *A. isoporum* and *A. ictaluri* could be reconciled, it would then be possible to eliminate to a large degree the confusion that has seemed beyond solution. There are isolated families of trematodes that may prove eventually to be closely related to groups that may emanate from the allocreadioid complex when that problem is resolved. What is hoped may be a step in that direction is made with the following proposals:

OPECOELOIDEA n.superfam.

DIAGNOSIS. Distome trematode parasites occurring in the intestine of freshwater and marine fishes. Suckers well developed; ventral sucker varying in position from well anterior to mid-level to posterior end of body, often embedded in a protruding fleshy lobe, stalk, or disk. Prepharynx short, if present;

pharynx well developed; cæca simple, extending well toward posterior end of body, sometimes with separate anal openings posteriorly, uniting and having a single such opening, or joining excretory vesicle and thus forming uroproct with excretory pore serving as an anus. Cuticle always unarmed but often thick and wrinkled; cercarial eye-spot pigment absent. Genital pore anterior to ventral sucker, often far so and to left of median line, rarely submarginal or dorsal. Cirrus sac present or absent and then sometimes with an accessory sucker near genital pore. Seminal vesicle unipartite, usually long and coiled within cirrus sac; external seminal vesicle absent. Testes two or rarely multiple, intercæcal, tandem, diagonal or even side by side in short-bodied forms. Ovary pretesticular, seminal receptacle if present an enlargement of Laurer's canal; vitellaria well developed and extensive, with follicles usually along most of body length. Uterus pretesticular, metraterm independent of cirrus sac and not especially thickened or otherwise modified. Eggs large, operculate, and occasionally with antopercular knobs or long filaments. Excretory vesicle sac-shaped or tubular, variable in length, sometimes extending almost to intestinal bifurcation but usually not anterior to ovary. Main excretory tubule on each side extends anteriorly a variable distance but never to pharyngeal level and becoming recurrent before receiving two secondary tubules, one extending anteriorly and one posteriorly. Each secondary tubule serves two groups of flame cells so that the excretory formula is $2[(n+n)+(n+n)]$; often $n=2$ in the cercaria and remains unchanged in the adult. Cercaria distome and cotylomicrocercous, i.e. tail short and consisting of a cup with glands in the wall or a glandular core and used for attachment to substratum. Eye-spots absent, cuticle without spines but often thick and with sensory papillae and 'hairs'. Simple or multi-pointed stylet set vertically in oral sucker. Cephalic glands in forebody with ducts on each side in one or two bundles, opening near tip of stylet. Excretory vesicle sac-shaped and thick-walled, confined to hindbody and sometimes with apex overlapping ventral sucker or deflected to its side. Excretory pores at junction of body and tail. Cercariae develop in sausage-shaped sporocysts in prosobranch gastropods; metacercariae in invertebrates or rarely fishes. Includes the families Opecoelidae and Opistholebetidae.

Family Opecoelidae Ozaki

Diagnosis. With the characters of the superfamily. Post-oral ring and pigment spots absent. Excretory pore at posterior end of body, ventral sucker always well removed from that end. Flame cells with a single cone of cilia. Anal opening(s) often present. Common parasites of both fresh-water and marine fishes. For subfamilies and genera see Manter (1947).

Family Opistholebetidae Travassos

Diagnosis. Muscular, thick-bodied trematodes with the characters of the superfamily. Mature specimens usually with conspicuous pigment spots concentrated near vitellaria. Ventral sucker from near mid-level to posterior end of body, with or without a surrounding disk that may be glandular, and joined by powerful muscles extending into the parenchyma. Oral sucker with a post-oral ring, pharynx

massive, oesophagus short or absent, ceca wide and never with ani or joining excretory vesicle to form uroproct. Uterus with coils slightly posterior to ovary, seminal receptacle always present. Excretory pore from posterior to dorsal in position, depending on location of ventral sucker. Flame cells sometimes and perhaps always complex with numerous cones of cilia per cell; formula $2[(2+2)+(2+2)]$ where known. Intestinal parasites of marine fishes, largely of the puffer group and species of *Calamus*. Includes the genera *Opistholebes* Nicoll (type), *Maculifer* Nicoll, *Heterolebes* Ozaki and *Pachycreadium* Manter. See Ozaki (1937) for diagnoses of genera other than *Pachycreadium* which is characterized by Manter (1954) and here emended to include the presence of a post-oral ring, and, in one of its species at least, complex flame cells with the formula given above for the family.

SUMMARY

Opistholebes diodontis n.sp. is described from the intestine of the porcupine fish, *Diodon hystrix*, and development from the metacercaria to the adult is outlined. In that process, the ventral sucker shifts from near the mid-level to the posterior end of the body and other rearrangements occur as a result of differential growth. Hence in the Opistholebetidae and probably the Gyliauchenidae and Cephaloporidae as well, the amphistome plan is considered to be secondary in phylogeny. None of those families belongs in the Paramphistomoidea. Instead, each is believed to be a distinct family of aberrant trematodes derived from a separate line of more generalized distomes. Representing one such line of descent are the Opistholebetidae and Opecoelidae which are more closely related to each other than either is to any other group. Hence a superfamily, Opecoeloidea, is proposed to include the two families and set them apart as a natural group distinct from the remainder of the allocreadioid complex which is discussed. The genus *Pachycreadium* is transferred to the Opistholebetidae, family diagnoses are revised, and a key to the species of *Opistholebes* is given.

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PARASITIC COPEPODS OF FISH FROM CEYLON. IV

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(With 14 Figures in the Text)

This contribution is intended to be the last of this series. As one who may claim to have revived interest in these crustaceans from this region since the days of Bassett-Smith just prior to the beginning of the century, I find it particularly gratifying that several Indian zoologists are now steadily engaged in adding to our knowledge of these forms. Altogether some forty-three genera containing about 120 species have now been recorded from the Indian Ocean. This must form only a small fraction of the total number of forms that actually occur on the wide variety of hosts inhabiting this region.

This paper deals with six species, three of which are new. The female of *Caligus constrictus* Heller is made known for the first time; this species and *Lernanthropus trifoliatus* Bassett-Smith are recorded since they were originally described and serve to supplement the original accounts. *Naobranchia lizae* (Kroyer) is recorded from this region for the first time and on a new host.

This paper was prepared for publication during a short stay at the Laboratory of the Marine Biological Association, Plymouth. I am grateful to the Director for the facilities provided.

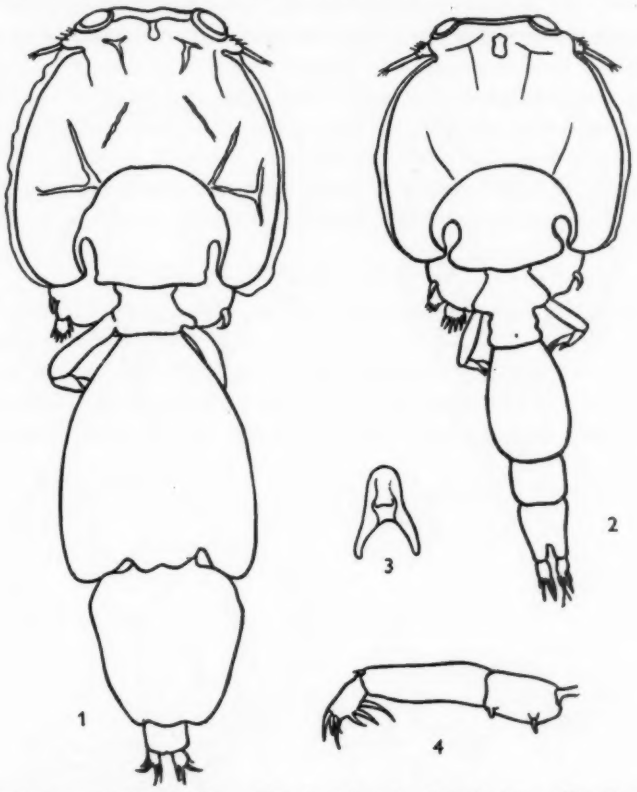
(1) **Caligus constrictus** Heller

Host and record. Numerous specimens of both sexes were found on the gills of *Alectis indicus* at Colombo.

Description of the female (Fig. 1). Cephalothorax as long as broad, a little more than one-third the entire length; frontal plates well defined; lunules prominent, widely separated from each other; eyes inconspicuous; median lobe, as wide as the lateral lobes taken together, not projecting beyond the latter. Free thoracic segment short, narrower than the median lobe of the cephalothorax. Genital segment only a little shorter than the cephalothorax, flask-shaped, extending posteriorly into short but distinct lateral lobes. Abdomen shorter than the genital segment, two-segmented; first segment broad, though not quite as broad as the genital segment, more than four times as long as the cylindrical second segment; anal laminae small, tipped with four setae, seta on outer margin smaller than the three setae on the posterior margin. Terminal joint of first antenna slender; second antenna hook-like; first maxilla a short spur and second maxilla a two-pronged spine; mouth-cone short; first maxilliped with two terminal spines; the strong claw formed by the second maxilliped with a small blunt spine arising from the ventral surface of its basal joint; furca short and tapering; the four pairs of swimming legs of the usual pattern; rudimentary fifth and sixth legs on ventral side of the lateral lobe of genital segment not visible in dorsal view.

Total length 6 mm.

Description of the male (Fig. 2). Cephalothorax as in the female but a little longer in proportion to the rest of the body. Free thoracic segment comparatively longer than in the female. Genital segment ovate, narrow anteriorly. Abdomen two-segmented, about as long as the genital segment but narrower; first segment a



Figs. 1–4. *Caligus constrictus* Heller. Fig. 1. Female, dorsal view. Fig. 2. Male, dorsal view. Fig. 3. Furca. Fig. 4. First swimming leg of male, ventral view.

little shorter than second; anal laminae and setae as in the female. First swimming leg bears a vestige of the endopodite in addition to a spine on the basal segment; other appendages as in the female.

Total length 5 mm.

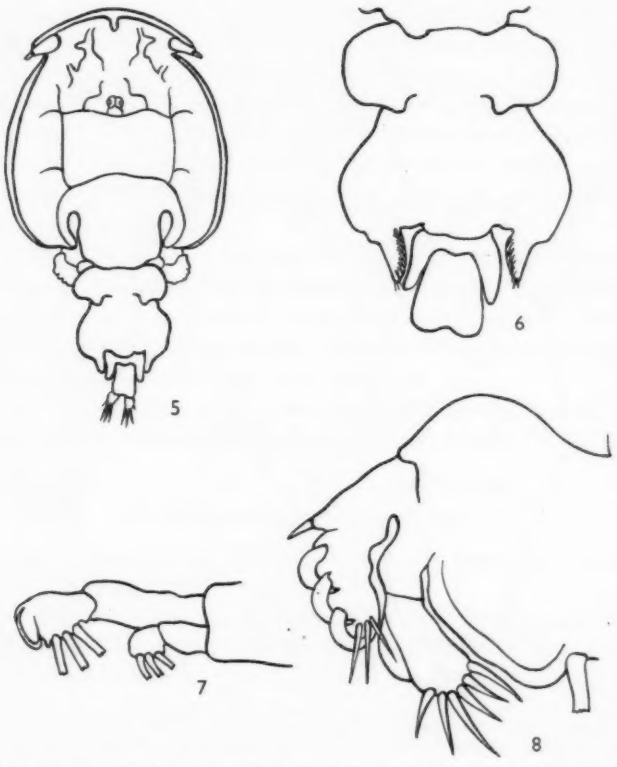
Remarks. Heller (1865) established this species on male specimens only, found on the gills of a *Stromateus* sp. in the Indian Ocean. This is, therefore, the first description and record of the female, and *Alectis indicus* is a new host for this species.

(2) **Alebion megacephalus** n.sp.

Host and record. A single female found on the skin of a carcharinid shark off the south-west coast of Ceylon is made the type of this new species.

Description of the female (Fig. 5). Cephalothorax ellipsoidal, longer than wide,

more than half the entire length; median lobe much wider than the lateral lobes; transverse grooves prominent, those of the lateral areas nearly equally spaced; eyes distinct, situated at about a third of the length of the cephalothorax from its anterior margin, and fused in the middle line. Free thoracic segment about as wide as the median lobe of the cephalothorax, its dorsal plates semicircular with small posterior lobes overlapping the genital segment. Genital segment as wide as the



Figs. 5–8. *Alebion megacephalus* n.sp. Fig. 5. Female, dorsal view. Fig. 6. Free thoracic, genital and abdominal segments enlarged, dorsal view. Fig. 7. First swimming leg, ventral view. Fig. 8. Third swimming leg, ventral view.

free thoracic segment but about twice as long, its lateral margins convex and unarmed with spines but its posterior processes with a row of spines on the inner margin and three spines at the tip. Abdomen two-segmented; basal segment much shorter than the terminal, with posterior processes extending on either side of the latter to about half way along it; terminal segment nearly cylindrical, stouter posteriorly; anal lamina tipped with three plumose setae and a shorter seta on the outer margin. First antenna two-jointed, terminal joint tipped with one or two short, spine-like setae; second antenna with a stout basal joint and a terminal claw; mouth-cone somewhat elongated; first maxilliped with a bifid terminal claw;

second maxilliped with a strong spine on the lower side of the terminal claw so that this appears to consist of two prongs; swimming legs of the usual pattern in the genus with the fourth pair reduced to short stumps.

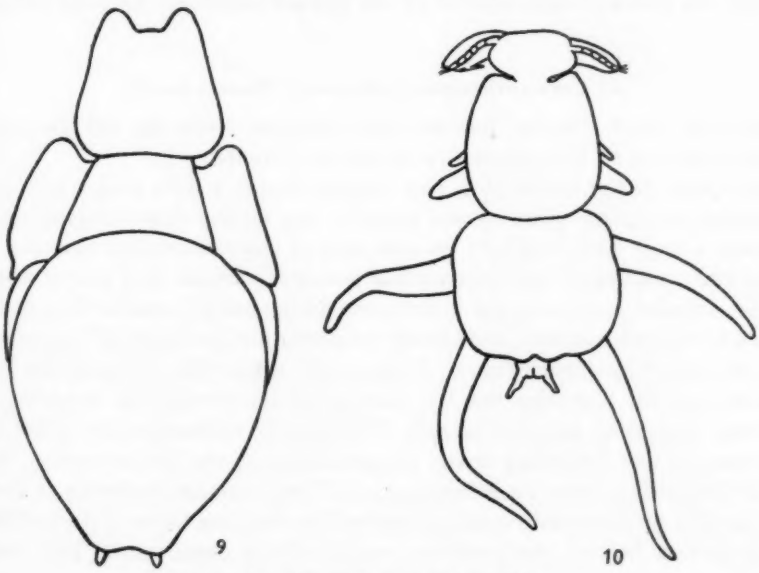
Measurements (in mm.): total length 5; cephalothorax 3·1; free thoracic segment 0·4; genital segment 0·7; abdomen and anal laminae 0·8.

Remarks. This species is characterized both by the length of the cephalothorax in proportion to the rest of the body and by the short posterior processes of the genital segment which scarcely reach half way along the abdomen, stopping short even of the posterior processes of the first abdominal segment. These two features, together with the shape of the dorsal plates on the free thoracic segment, distinguish this species from all others described by Wilson (1932) as well as from the more recently described *A. alatus* Gnanamuthu (1951) from the Indian Ocean.

(3) **Lernanthropus priacanthi** n.sp.

Host and record. Several specimens of both sexes were found on the gill filaments of *Priacanthus hamrur* at Colombo.

Description of the female (Fig. 9). Cephalothorax trapezoidal, narrow in front, with large, ventrally produced lateral flaps; it is separated from the body by a neck-like region. Body dorsally convex, covered by a plate divided into anterior



Figs. 9, 10. *Lernanthropus priacanthi* n.sp.

Fig. 9. Female, dorsal view.
Fig. 10. Male, dorsal view.

and posterior regions by a shallow transverse groove; anterior region short, provided with wing-like lateral expansions; posterior region as broad as the anterior but much longer, ovate, narrowing behind; hindmargin of dorsal plate

slightly concave. Third pair of legs flattened into broad laminae parallel with the ventral surface and covering nearly the whole of it; laminae of the fourth legs broad, covered for the most part by the laminae of the third legs, and either not projecting beyond the hindmargin of dorsal plate or with only the tips of their exopodites extending beyond the hind margin of dorsal plate.

Total length 3·7 mm.

Description of the male (Fig. 10). An oval head region, nearly twice as wide as long, is separated from the thorax by a neck-like constriction; cephalothorax much longer than wide and longer than the hindbody; a rectangular dorsal plate covers the genital segment and the single rami of the third and fourth legs extend freely outwards from its antero-lateral and postero-lateral regions respectively; hindmargin of dorsal plate slightly concave in the middle line; abdomen with its tapering anal laminae visible in dorsal view.

Total length (not including the legs) 1·8 mm.

Remarks. If the female only of this new species is considered, it is not readily distinguishable from that of *Lernanthropus larvatus* Heller, found on a closely related host, *Priacanthus ocellatus*, in the Indian Ocean. The males, however, of the two species are very different, especially in the shape of the dorsal plate and the nature of the third and fourth legs. The minor differences between the females of these two forms are accentuated by the marked differences between the males.

(4) **Lernanthropus trifoliatus** Bassett-Smith

Host and record. Several females were obtained from the gill filaments of *Caranx kalla* and *C.* (*Carangoides*) *praeustus* at Colombo.

Description of the female (Fig. 11). Cephalothorax a little longer than broad, trapezoidal in outline when viewed dorsally; the folding downwards of its sides produces a large lobe anteriorly on each side of the well-marked antennal area. Dorsal plate marked off into anterior and posterior portions by a pair of marginal sinuses; anterior portion narrow in front and flaring behind, smaller than posterior portion being both narrower and shorter; posterior portion large and almost round, its hind edge faintly emarginate. Dorsal plate completely covering the genital segment and the abdomen but the laminae of the fourth legs extending considerably beyond its posterior margin. Third pair of swimming legs folded in the usual manner and projecting nearly at right angles to the ventral surface; fourth legs divided to their base, rami flattened into broad laminae narrowing at the tips; fifth legs also produced into a pair of laminae shorter than those of the fourth legs, not projecting beyond the posterior margin of the dorsal plate; anal laminae lanceolate, ending short of the laminae of the fifth legs.

Remarks. This species was described from female specimens found on the gills of *Polynemus tetradactylus* at Bombay. The type specimen was 8 mm. long. The present specimens are only 3·7–5·55 mm. long; they are without egg-strings and so may not be full grown. Bassett-Smith (1898) evidently mistook the laminae of the fifth pair of legs as belonging to the fourth pair and so gave the name *trifoliatus* to the species.

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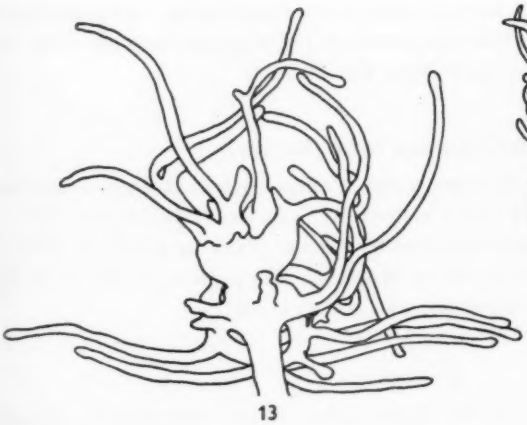

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Fig. 11. *Lernanthropus trifoliatus* Bassett-Smith. Female, dorsal view.

Figs. 12–14. *Lernaeenicus ramosus* n.sp. Fig. 12. Female, ventral view. Fig. 13. Dorsal view of head region of female. Fig. 14. Ventral view of head region of another female.

(5) **Lernaeenicus ramosus** n.sp.

Host and record. Seven females were collected in March 1948 and two more in February 1954, all of them from the same host fish *Epinephelus morrhua* from the Wadge Bank off Ceylon. The parasites were attached at various sites on the body of the fish.

Description of the female (Fig. 12). Head in line with thorax and roughly hemispherical in shape, its ventral surface flattened; attachment pads in paired groups on ventral surface, one group on either side of the mouth and a pair of pads farther back; horns in two sets, one set consisting of three pairs of branched horns arising from the anterior, antero-lateral and postero-ventral regions of the head, and the other set consisting of a pair of much-branched horns arising from the thorax dorso-laterally and curving backwards and downwards below the base of the neck. Branches of the horns not radiating out in all directions but lying nearly in the same plane between the body muscles and skin of the host; the main horns constant in number but their branches not symmetrically developed on the two sides even of the same individual. Neck narrow, cylindrical, passing imperceptibly into the stouter trunk (genital segment) region. Abdomen well marked from the trunk and gradually tapering to its extremity. Egg-strings narrow, longer than abdomen.

Total length 18 mm. Head 2 mm., neck and trunk 10 mm., abdomen 6 mm.

Remarks. The most striking feature of this parasite is its complex system of horns. *Lernaeenicus polyceraus* Wilson (1917) and *L. gnathonicus* Leigh-Sharpe (1934) are two other species with branched cephalic and thoracic horns, but in neither of them are the horns so large or so profusely branched. The long-necked *L. polyceraus* is very distinct from the short-necked *L. gnathonicus* and *L. ramosus*, and these two species are distinguished from each other by the length of the abdomen relative to the trunk; in *L. gnathonicus* the abdomen is one-fifth longer than the trunk, but in *L. ramosus* the abdomen is shorter than the trunk.

(6) **Naobranchia lizae** (Kroyer)

Host and record. Numerous females, some of them with associated males, were found on the gill filaments of *Pellona hoevenii* at Colombo in February 1954.

Remarks. This species has been recorded by Kroyer (1863) and Wilson (1915) off the Atlantic coast of North America on *Mugil* sp. The present record is the first from the Indian Ocean and *Pellona hoevenii* is a new host.

ERRATUM

In the previous paper of this series (Kirtisinghe, 1950) I described a parasitic copepod from the gills of *Psettodes erumei* and called it *Protochondracanthus psettodis* n.g., n.sp. Since then I have found that this form, taken probably from *Uranoscopus scaber* in the Mediterranean, had been described by Heller (1865) and named *Chondracanthus alatus*. Bassett-Smith also noted this parasite on the gills of *Psettodes erumei* at Bombay and identified it as Heller's species, *Chondracanthus*

alatus. I had not seen this passing reference by Bassett-Smith, and Heller's description and figures are inextricably confused with another species he was describing at the same time. However, his figure of the entire animal leaves no doubt that *Protochondracanthus psettodis* Kirtisinghe is a synonym of *Chondracanthus alatus* Heller.

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ANATOMY AND HISTOLOGY OF THE ALIMENTARY TRACT OF THE FEMALE OF THE BITING MIDGE *CULICOIDES NUBECULOSUS* MEIGEN (DIPTERA: HELEIDAE = CERATOPOGONIDAE)

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(With 18 Figures in the Text)

INTRODUCTION

The adult blood-sucking females of a number of species of *Culicoides* are pests in many parts of the world. The irritation and annoyance caused by their attacks on man and his animals are often so great that they constitute a serious problem. The chief significance, however, of *Culicoides* spp. is that they may act as vectors of filarial and virus diseases to man and his animals (see Hill, 1947). *C. nubeculosus*, in particular, was found by Steward (1933) to be the vector of *Onchocerca cervicalis*, which infests the cervical ligament in horses and is the principal cause of nuchal disease (fistulous withers and poll-evil) in England. It is also incriminated by Moignoux (1952) as the vector of *O. reticulata*, which infests the suspensory ligament of the pastern joint of horses in the south of France.

In view of the economic importance of *Culicoides* spp. as pests and as vectors of disease, much attention is being paid to their study in many parts of the world. Hitherto, however, no attention has been paid to the study of the alimentary tract, especially that of the female, in any species. To my knowledge, the references on this subject are confined to a figure of the alimentary tract of *C. kiefferi* (plate XXII), an elementary account of the Malpighian tubules given by Patton & Cragg (1913), and some brief class-notes and figures of the alimentary tract and the salivary glands (pp. 135, 140, 149) of *C. varius* (?) (probably *C. obsoletus* Mg., syn. *Ceratopogon varius* Winnertz, 1852, *Linn. Ent.* **6**, 35; see Edwards, 1939, p. 143) given by Patton & Evans (1929).

Since the alimentary tract constitutes the environment of the ingested parasites, it has therefore a special importance and for this reason it has been studied in many blood-sucking flies. The present work attempts to extend our knowledge to the alimentary tract of *Culicoides* and to relate this to the previous studies on other biting flies. *C. nubeculosus* was chosen for this study because it is the only species which, hitherto, has been maintained as a laboratory culture, so that adults can be obtained at intervals throughout the year. As males are of no importance as vectors of diseases, the study was confined to the females.

MATERIAL AND TECHNIQUE

The flies were reared in the laboratory as is described by Megahed (1955).

(1) *Dissections.* Dissections were made on freshly killed specimens. The flies were dissected piecemeal in 0·65 % saline solution, and by means of a pair of fine sewing-needles mounted in pin-holders.

(2) *Sectioning.* Whole insects were used in sectioning because of their small size. A cytological study was not intended, and it was only necessary to use a fixative which gives a good histological picture. Picric acid fixatives were used, but they caused the specimens to shrink and symmetrical sections were found difficult to obtain. This disadvantage was overcome by killing and preserving the specimens in warm Pampel's fluid before the picric acid fixative was used, as is recommended by Abul-Nasr (1950). Ester-wax was used as an embedding medium. The wings and legs of the preserved specimens were removed, and the trimmed insects were transferred to Duboscq-Brazil and left for 24 hr. The material was then washed and left in 70 % alcohol for 24 hr. (two to three changes), transferred to 1 : 1 mixture of 70 % alcohol and cellosolve and left overnight. Clearing in pure cellosolve for 1–2 days (two to three changes) followed. The cleared material was then transferred to 1 : 1 mixture of cellosolve and ester-wax for 2½ hr. in an oven at 48° C. Two baths of pure ester-wax in the oven, for 1½ hr. each, followed. Finally, each specimen was embedded in pure ester-wax in a watch-glass. Sagittal, horizontal and transverse sections were cut at 6μ thick. Ehrlich's haematoxylin, Heidenhain's haematoxylin and Mayer's haemalum as nuclear stains, and eosin in absolute alcohol, orange G, and Van Gieson as counterstains were tried. Ehrlich's haematoxylin and eosin in absolute alcohol gave satisfactory results and were used throughout the work.

General

The alimentary tract of female *C. nubeculosus* (Fig. 1) is a simple tube. The fore-gut consists of the sucking apparatus (in part), the oesophagus (*oes.*), the oesophageal valve (*oes.v.*) and the oseophageal diverticulum (*oes.d*). The mid-gut consists of two portions, an anterior narrow portion (*ant.m.g.*) and a posterior dilated one (*st.*). The hind-gut consists of an anterior tubular portion which cannot be differentiated into parts (*s.int.*), and a posterior dilated rectum (*rec.*) which contains two rectal papillae (*rec.p.*).

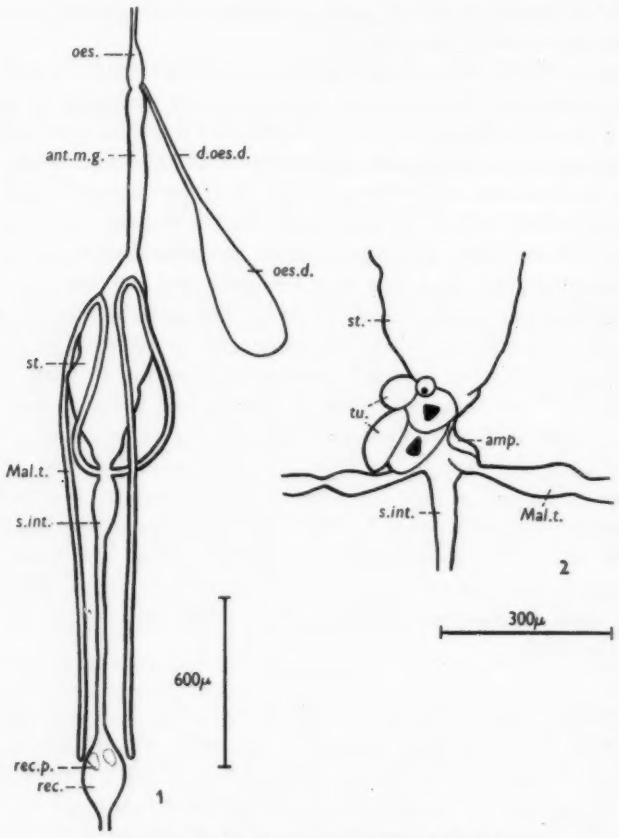
In addition, there are two sets of structures associated with the alimentary tract, namely, the salivary apparatus and the Malpighian tubules (*Mal.t.*).

THE FORE-GUT

The sucking apparatus

The sucking apparatus consists of two chambers or pumps contained within the head-capsule. In Nematocera, the nomenclature of these two pumps has been the subject of much controversy. Snodgrass (1943), however, shows clearly that the first pump is the homologue of the preoral cibarial pocket of the orthopteroid insects and terms it the 'cibarial pump', and that the second pump, which he terms the 'pharyngeal pump', is a modification of the pharynx.

In particular, the sucking-apparatus of *C. nubeculosus* resembles that of *C. pulicaris* (Jobling, 1928) and that of *C. impunctatus* (Gad, 1951). But Jobling termed the cibarial pump the 'pharynx' and the pharyngeal pump the 'oesophageal



Figs. 1, 2. *Culicoides nubeculosus* Mg. (female).

Fig. 1. The alimentary tract—diagrammatic. (The sucking apparatus and the salivary apparatus are not shown.)

Fig. 2. Tumours at the base of the stomach, and abnormal features in the Malpighian tubules.

amp.	ampulla	*oes.d.*	oesophageal diverticulum
ant.m.g.	anterior segment of the mid-gut	*rec.*	rectum
d.oes.d.	duct of the oesophageal diverticulum	*rec.p.*	rectal papilla
		s.int.	small intestine
Mal.t.	Malpighian tubule	*st.*	stomach
oes.	oesophagus	*tu.*	tumours

pump', and Gad did not mention the anterior and the posterior sphincters of the pharyngeal pump.

According to Snodgrass (1943), the cibarial pump is present in all the Diptera. The Brachycera also have a pharyngeal pump, but it is formed from the anterior

part of the pharynx and is activated by the precerebral dilator muscles; the Cyclorrhapha have only the cibarial pump.

The oesophagus

The oesophagus (*oes.*, Fig. 3) is a thin-walled, narrow and short tube. It is about 225μ long from its beginning at the posterior end of the pharyngeal pump to the anterior limit of the mid-gut. It is very narrow anteriorly, but it expands gradually to become about 36μ wide in its widest portion. It proceeds from its point of origin just behind the occipital foramen backwards through the cervix and joins the mid-gut about the level of the forelegs.

The wall of the oesophagus consists of a layer of ill-defined epithelial cells (*epi.c.*), which differ in shape from low cubical to flat, according to the degree of contraction or expansion of the oesophagus. In the anterior portion of the oesophagus the nuclei of the cells are somewhat big and are oval in shape, but posteriorly they are round and rather small. The epithelial layer rests on a delicate basement membrane, followed by a double layer of internal longitudinal muscle fibres (*l.m.*) and external circular muscles (*c.m.*). The cuticular intima (*cut.int.*), which lines the inner surface of the epithelial cells, is thin. It is easily detected anteriorly where it is of a dark colour and has a serrated appearance, but it becomes so thin and colourless posteriorly that it becomes difficult to detect in most sections.

In *Simulium nigroparvum* (Cox, 1938) there is only a layer of circular muscle fibres. *In Phlebotomus papatasii* (Adler & Theodor, 1926) the oesophagus is attached for a considerable distance to the sides of the pharyngeal pump and thus a pouch is formed between the external wall of the pharyngeal pump and the oesophagus; this pouch has been found to contain *Herpetomonas*. In the mosquito (Nuttall & Shipley, 1903) the oesophagus is plentifully supplied with bands of muscles, and the average outline of the cells is roughly cubical. In *Tabanus* (Cragg, 1920) the cuticular intima is well developed.

The oesophageal valve

The posterior end of the oesophagus is invaginated into the anterior portion of the mid-gut to form a regulatory valve—the oesophageal valve (*oes.v.*, Fig. 3). The oesophageal wall extends for a short length (about 27μ) into the lumen of the mid-gut (*m.g.*), then is reflected forwards to join the anterior end of the latter. It consists of a single layer of small but well-defined cubical cells (*epi.c.*) with small round nuclei. The stomodaeal circular muscles around the invagination are highly developed to form the sphincter muscles (*sph.m.*), occluding the lumen by their contraction. No cuticular intima can be detected.

The oesophageal valve has almost the same structure in the nematoceran and brachyceran blood-sucking flies. In these flies the oesophageal valve has no role in the formation of the peritrophic membrane, and seems to serve only to regulate the influx of the ingested fluid into the mid-gut, and to prevent its regurgitation. On the other hand, in the cyclorrhaphan blood-sucking flies, e.g., *Glossina* (Wigglesworth, 1929), in which the peritrophic membrane is produced by a group

of specialized cells at the anterior end of the mid-gut, the oesophageal invagination is funnel-shaped and is composed of large cells forming a solid plug, and serves to press the precursor fluid of the peritrophic membrane into a solid homogeneous tube. Smart (1935) states that in *Simulium ornatum*, the component tissues of the

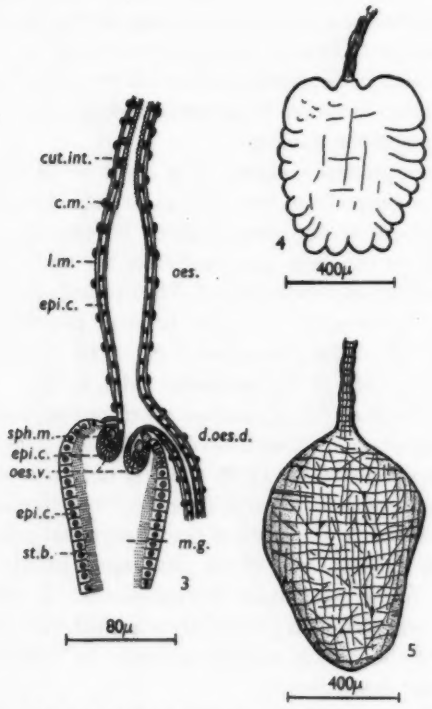


Fig. 3. A diagrammatic median longitudinal section through the oesophagus and the anterior segment of the mid-gut.

Fig. 4. A diagram of a distended oesophageal diverticulum showing one of the various shapes taken by it during peristalsis.

Fig. 5. A diagram of a distended oesophageal diverticulum showing the arrangement of its muscles.

c.m.	circular muscle	*m.g.*	mid-gut
cut.int.	cuticular intima	*oes.*	oesophagus
d.oes.d.	duct of the oesophageal diverticulum	*oes.v.*	oesophageal valve
		sph.m.	sphincter muscles
epi.c.	epithelial cells	*st.b.*	striated border
l.m.	longitudinal muscle		

oesophageal valve, except for some ill-defined differences in the shape and size of the component cells and the complete absence of a striated margin in the cells, are similar to those of the anterior portion of the mid-gut. In *S. nigroparvum*, Cox (1938) states that the inability to distingnish a cuticular intima made it difficult to determine the limits of the fore- and the mid-gut, i.e. of the ectodermal and meso-

dermal tissue. In *Culicoides nubeculosus*, however, the cells of the valve can be easily differentiated from those of the anterior portion of the mid-gut. They are obviously smaller and narrower, their nuclei are smaller and they lack the characteristic striated border (*st.b.*). In *Phlebotomus papatasii*, Adler & Theodor (1926) state that a small pouch forms between the sides of the anterior portion of the mid-gut and the oesophageal valve. Four small elliptical oseophageal glands, which contain yellow granules, lie round the commencement of the mid-gut, and each gland opens by a minute duct into the pouch between the oesophageal valve and the mid-gut.

The oesophageal diverticulum

Just in front of the point of invagination of the oesophagus into the mid-gut, the ventral wall of the oesophagus is evaginated to form a single capacious dumb-bell-shaped diverticulum (*oes.d.*, Fig. 1), which lies ventral to the mid-gut, with some inclination to the left when distended with fluid (in some specimens at least). The oesophageal diverticulum consists of a dilated sac with a long narrow duct (*d.oes.d.*, Fig. 1): the sac is capable of much distension, while the duct is far less capable of distension. When empty, the diverticulum is collapsed and lies in the thorax or, sometimes, may extend into the anterior end of the abdomen. When distended with water or sugar solutions or raisin sap, it extends into the abdomen to a level which varies according to the degree of its distension and may reach the 4th or the 5th abdominal segment. When the diverticulum contains a small amount of fluid, this collects at the bottom of the diverticulum while the rest of its length becomes very contracted and collapsed. On the other hand, when the diverticulum is highly distended the contracted portion becomes relatively short.

When dissected out in saline solution, the diverticulum is seen to undergo vigorous peristaltic movements which are best demonstrated in the distended diverticulum. In the latter case, the movements are seen to follow one another from one side to the other and from the fundus upwards, and vice versa (thus ensuring a thorough churning of the contained fluid). During peristalsis, however, the outline of the distended diverticulum becomes very variable and takes on many curious shapes (e.g. Fig. 4).

The oesophageal diverticulum is a very thin-walled structure. In sections, when the diverticulum is empty, it is so collapsed that its wall only appears as a wrinkled membrane with a few scattered small nuclei. On the other hand, when it is distended only a very thin outline can be seen with a still thinner cuticular intima. In favourable sections of the anterior portion of the duct of the diverticulum (*d.oes.d.*, Fig. 3), however, it is seen to be of the same structure as that of the oesophagus. The musculature of the dilated part of the diverticulum was studied by using the method of Nuttall & Shipley (1903), i.e. by simply allowing the dissected diverticulum (distended) to dry outwardly on a slide and then staining with aniline or other stain; eosin in water was found satisfactory. It consists of a network of muscle fibres, the longitudinal and the circular fibres are incomplete. There are also diagonal interlacing fibres. These muscle fibres are seen to be branches of small muscle bands (Fig. 5). Such an arrangement of the muscle

fibres is responsible for the vigorous peristaltic movements and the variable appearance of the distended diverticulum.

Adler & Theodor (1926) state that in female *P. papatasii* there is a very narrow sphincter at the junction of the oesophagus and the diverticulum; the diverticulum opens into the oesophagus at a varying distance from the posterior end of the latter, and exceptionally it opens into the pharyngeal pump together with the oesophagus. I was unable to detect such a sphincter in *Culicoides nubeculosus*, and it seems that the diverticulum opens into the oesophagus by means of a simple pore as is the case with the ventral diverticulum in mosquitoes (Nuttall & Shipley, 1903). It was observed, however, that when the dissected diverticulum is distended with fluid, the upper undistended part (sometimes long, sometimes short, according to the degree of distension) is extremely contracted, and when the contracted part of the diverticulum is excised anywhere above this level the contained fluid does not flow out.

It is noteworthy that in sections of some females the lumen of the oesophageal diverticulum was seen to contain fungal hyphae. The spores of the fungus must have been ingested with the raisin sap and have developed within the diverticulum.

All the studied blood-sucking flies, except mosquitoes, have only one oesophageal diverticulum, which is more or less similar to that of *C. nubeculosus*. In *Tabanus* (Cragg, 1920) it is bilobed. In mosquitoes (Nuttall & Shipley, 1903) there are three diverticula: one large and ventral, which is similar to that of *C. nubeculosus*, and two small latero-dorsally situated ones which open laterally into the oesophagus.

THE MID-GUT

The mid-gut, or ventriculus, of *C. nubeculosus* is a simple straight tube of varying diameter. It extends from the base of the outer fold of the oesophageal valve in the anterior part of the thorax, about the level of the posterior limit of the bases of the forelegs, to the point of origin of the Malpighian tubules about the middle of the 5th abdominal segment. The relative position with regard to the abdominal segments, however, varies according to the degree of distension of the gut with food. It consists of two portions which merge into one another: an anterior narrow portion, and a posterior portion which is several times wider. At its posterior end the gut narrows abruptly, to form the mesenteric sphincter (ventricular valve), before it joins the hind-gut.

The anterior portion of the mid-gut, which in related insects is termed the 'cardia' by some authors (e.g. Cragg, 1920; Adler & Theodor, 1926), is here termed the anterior segment (*ant.m.g.*, Fig. 1). The term cardia should be restricted to the anterior end of the ventriculus (Snodgrass, 1935, p. 361). According to the same author (p. 360), nearly all the students of the alimentary canal of Diptera have called the cardia the 'proventriculus', but its true nature is shown by the fact that the stomodaeal valve is invaginated into its anterior end. In female *C. nubeculosus*, however, the cardia is less differentiated and is clearly the anterior end of the mid-gut. The posterior segment of the mid-gut, as in related insects (e.g. mosquitoes, *Phlebotomus papatasii*, *Simulium*, *Tabanus*, etc.), is here termed the

stomach (*st.*, Fig. 1). When empty, it has a tubular shape, but more usually it takes on an ovoid shape, due partly to traction during dissection and partly to its containing some fluid.

The anterior segment is widest anteriorly, especially at the cardia. It extends to the posterior end of the thorax (or the anterior end of the abdomen), where it merges into the stomach. It does not become distended with blood, in marked contrast with the stomach. In sections of flies fixed directly after a full blood meal, it is seen to contain varying amounts of blood but is never distended with it. When the gut is dissected out after such a meal, usually no more than a trace of blood can be seen in the anterior segment. This is due partly to traction during dissection and partly to the release of the skeletal pressure exerted against the stomach, resulting in the blood being driven from the anterior segment into the stomach.

The stomach is the chamber into which almost all the ingested blood is received, and it is capable of great distension. In flies not fed on blood it reaches the middle of the 5th abdominal segment, but when fully distended with blood it fills the greater portion of the abdomen, the rest of the viscera being pushed into the last two or three segments. Horizontal sections of the engorged flies show the stomach to be ovoid in shape. In dissections, the distended stomach assumes a globular shape, but this, of course, is due to the release of the skeletal pressure exerted against it.

Normally, the stomach lies about the middle line of the abdomen, but its position varies greatly according to the condition of the oesophageal diverticulum and the ovaries. When the diverticulum is distended and the stomach is empty, the portion of the latter situated above the diverticulum is pushed towards the dorsal vessel, and the rest of the stomach curves down and lies against the ventral nerve cord. When the stomach is distended with blood, the distension of the diverticulum apparently does not matter; the distension of the abdomen seems to cope with both of them. Lewis (1953) observed that in *Simulium damnosum*, $\frac{1}{2}$–1 hr. after a blood meal, the tubular part of the mid-gut though devoid of blood contained some gritty particles similar to those found in the oesophageal diverticulum. He suggests that the diverticulum under pressure from the expanding mid-gut may disgorge some of its contents into the oesophagus from which they pass into the mid-gut. I was unable to detect this in *Culicoides nubeculosus*, though it very probably occurs. When the ovaries are developed, they press the stomach against the ventral nerve cord.

The mid-gut shows peristaltic movements when dissected out in saline solution. This is most marked in the stomach, especially when it contains syrup or a small amount of blood. In horizontal sections of some flies fixed after a small meal of blood, the stomach was seen contracted about the centre, assuming the form of an hour-glass. A similar appearance was observed in the stomach of mosquitoes by Nuttall & Shipley (1903).

When it is empty, the surface of the mid-gut, and particularly that of the stomach, has a roughened and irregular appearance, due to the heaping of the epithelium into folds or villi.

The wall of the mid-gut (Figs. 6, 7) consists of a single layer of epithelial cells

which rests upon a delicate basement membrane (*b.m.*), and is surrounded by a network of inner circular (*c.m.*) and outer longitudinal muscles (*l.m.*). The mid-gut, and especially the stomach, is well supplied with tracheae, tracheoles and transition cells, which rest on the outer surface of the gut.

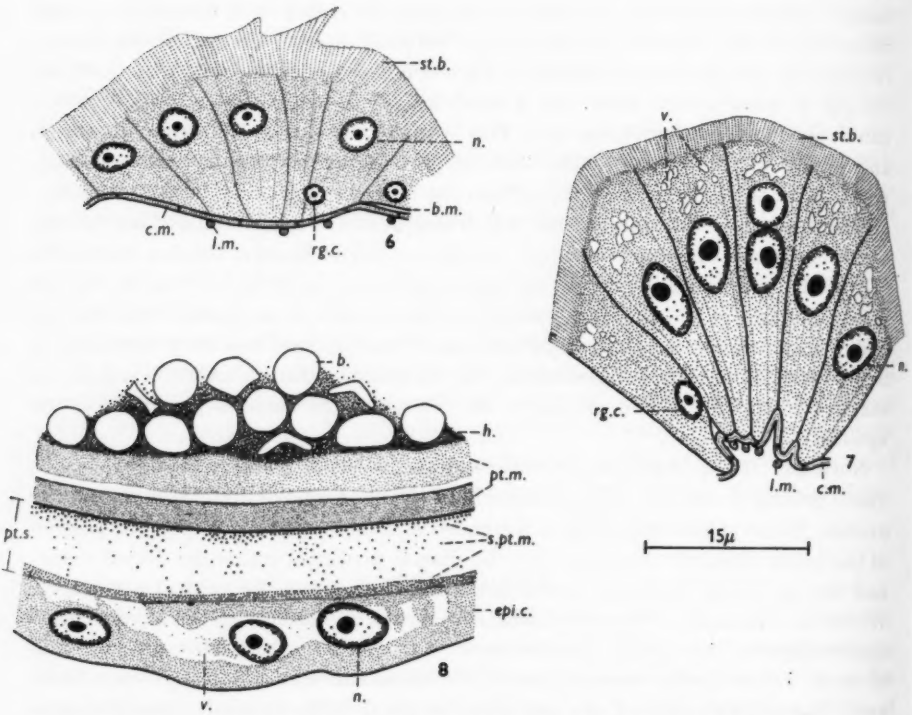


Fig. 6. Transverse section through the anterior segment of the mid-gut in an unfed fly less than 24 hr. old.

Fig. 7. Transverse section through the stomach in the same fly.

Fig. 8. Transverse section through the distended stomach of a fly 5 hr. after a full meal of blood—showing the peritrophic membrane.

b.	blood	*pt.s.*	peritrophic space
b.m.	basement membrane	*rg.c.*	regenerative cells
c.m.	circular muscle	*s.pt.m.*	droplets of secretion—the fluid precursor of the peritrophic membrane—secreted by the flattened epithelium of the stomach
epi.c.	epithelial cells		
h.	haematin		
l.m.	longitudinal muscle		
n.	nucleus	*st.b.*	striated border
pt.m.	peritrophic membrane	*v.*	vacuoles

The ventricular epithelium varies greatly according to functional activity. When the gut is empty, the cells are columnar or cubical in shape and are grouped into folds or villi, which project into the lumen and give it a stellate appearance in cross-sections. The depth of these villi, and consequently the width of the lumen,

is very variable among different specimens, but they are generally far higher in the stomach than in the anterior segment. After a full blood meal, the greater part of the length of the wall of the stomach suffers much distension, the epithelial cells flatten and the villi disappear (Fig. 8). When the fly is allowed a smaller meal, the villi become reduced or disappear totally, but the epithelial cells become more or less low cubical in shape. In the anterior segment, however, the villi do not disappear nor the cells flatten after a blood meal; the amount of blood in the lumen is always small and does not cause any distension of the anterior segment.

In the unfed flies, the epithelial cells differ in height and shape according to their situation. The cytoplasm is granular with a conspicuous fibrillar arrangement along the long axis of the cell. The nucleus (*n.*) is large, oval or round in shape, with a conspicuous nucleolus, and the chromatin granules are arranged mostly at the periphery. Each cell has one nucleus, but some cells, especially in the stomach, have two nuclei. The nucleus is usually situated towards the inner end of the cell, but in some cells it is central.

The striated border (*st.b.*) is evident in all the cells of the mid-gut. It stains pink with eosin. In the stomach it may disappear or become homogeneous due to the enormous vacuolation or flattening of the cells. The variable character of the striated border was observed in *Glossina* by Wigglesworth (1929, footnotes, pp. 291, 308), who states that it agrees with the observations of van Gehuchten upon the epithelium in the larva of *Ptychoptera* (Ptychopteridae, syn. Liriopeidae).

There are, however, some differences between the epithelium of the anterior segment and that of the stomach (cf. Figs. 6, 7). The cells of the anterior segment do not stain as well with haematoxylin as do those of the stomach, and are relatively small. Their nuclei are also smaller than those of the cells of the stomach and are usually round in shape, while those of the stomach are usually oval. The most marked difference, however, rests in the striated border. In the anterior segment, the striated border is almost always thick, sometimes homogeneous but never disappears except in occasional cells which are about to discharge vacuolated masses of cytoplasm. In the stomach it is always thin and may become homogeneous or totally disappears. Vacuoles are usually present in the cells of both segments, but in the stomach they are relatively big, in greater numbers and more usually are located at the inner end of the cells.

Scattered throughout the ventricular epithelium, there are small cells—the regenerative cells (*rg.c.*, Figs. 6, 7)—which occur singly. They are generally few in number and are more easily demonstrated in the epithelium of the anterior segment. They are usually seen near the basement membrane or wedged between the bases of the columnar cells. What can be readily seen of them is the nucleus, which is small. Their cytoplasm cannot be differentiated from that of the epithelial cells; in some instances, however, it may be seen as a clear area.

The muscular coat of the mid-gut is thin. It consists of an inner layer of circular fibres, which are in close contact with the basement membrane, and an outer layer of longitudinal fibres. This double layer of muscle fibres forms a network which adapts itself to the irregularities of the surface of the mid-gut into the hollows at the bases of the villi and also between the bases of adjacent cells (Fig. 7).

At its posterior end, just above the openings of the Malpighian tubules, the stomach narrows abruptly to form the mesenteric sphincter (*mes.sph.*, Fig. 9). The circular muscles of this region are exaggerated to form the sphincter muscles (*sph.m.*). The cells of the mesenteric sphincter seem to lack a striated border, but it is common to see them vacuolated, e.g. when the stomach is highly vacuolated. As in the tsetse flies (Lester & Lloyd, 1928), this sphincter serves a double purpose. It holds back the blood meal, allowing only waste products to pass, and in the act of opening it closes the openings of the Malpighian tubules so that faecal matter cannot enter them.

There seems to be but a little difference—probably specific—in the morphology and the histology of the mid-gut between *Culicoides nubeculosus* and the nematoceran and brachyceran blood-sucking flies. In *Simulium ornatum* (Smart, 1935), the epithelium of the stomach, when undistended, may be as thick as that of the anterior segment; the striated border is thicker in the anterior segment. In *Phlebotomus papatasii* (Adler & Theodor, 1926), the two segments of the mid-gut differ histologically; the cells of the stomach lack the striated border, while the cells of the anterior segment have a conspicuous striated border with the striae (rods) each 7·5–10μ long. In the mosquito (Christophers, 1901), there is little structural difference between the anterior segment and the stomach; Nuttall & Shipley, however, state that the mid-gut has a similar structure throughout. In *Tabanus* (Cragg, 1920), the layer of cells in the anterior segment is folded into innumerable small caeca; the striated border is remarkably well developed in the anterior segment, while it is delicate in the stomach. Cragg did not mention the mesenteric sphincter, but Wigglesworth (1931*a*) described it in *Chrysops silacea*.

On the other hand, in the cyclorrhaphan *Glossina* (Wigglesworth, 1929), the mid-gut is long and coiled, and is divided into three regions. The anterior segment is composed of an irregularly columnar epithelium which does not stain deeply with haematoxylin. The base of each cell is invaginated to receive the circular muscle fibres, a peculiar feature which is doubtless associated with the great distension to which this region of the gut is subjected; in this segment there is a zone of giant cells which contain symbionts. In the middle segment, the epithelial cells are heaped together so that in the contracted state the lining of the gut appears to be composed of many layers of cells packed with deeply staining granules and proceeding as a free border, sometimes hyaline, sometimes striated, which stains pink with eosin. The change from the middle to the posterior segment is less abrupt. After a few secondary turns the diameter of the gut diminishes, the epithelium gradually becomes less heaped and eventually gives way to a regular cubical or low columnar of rather deeply staining cells which continues unchanged until the entry of the Malpighian tubules marks the termination of the mid-gut.

The peritrophic membrane

The peritrophic membrane (*pt.m.*, Fig. 8) is a very thin capsule which envelops the ingested blood. It does not exist in the unfed female, and only forms in the stomach after a blood meal. When the blood is digested the membrane is evacuated with the excreta.

The peritrophic membrane is quite apparent in sections of flies fixed 5 hr. after a blood meal. In these flies the blood mass is seen separated from the epithelium, and the peritrophic membrane, which stains pink, attains a definite outline though not uniform in thickness nor, in some places, in texture.

In the anterior end of the stomach, which remains contracted, the space between the peritrophic membrane and the epithelium—the peritrophic space (*pt.s.*)—is considerable. The outline of the peritrophic membrane is irregular and shows the impression of the villi. Following this region—in the distended part of the stomach —the peritrophic membrane becomes thinner and the peritrophic space narrower. In the region above the mesenteric sphincter, the peritrophic membrane thickens again and the peritrophic space widens.

In some sections, the membrane is seen to be composed of a few successive layers or lamellae, the innermost of which is the oldest and is rather thick and brown in colour. In favourable places the epithelial cells were seen secreting droplets of various sizes (*s.pt.m.*, Fig. 8) which stain deep pink with eosin. These droplets stream towards the peritrophic membrane and add to its substance. It may be assumed that these droplets, or some of them, are digestive secretions. But the fact that they do not appear 24 hr. after the meal, while the stomach still contains many intact corpuscles, does not favour this assumption. In some places the outer surface of the peritrophic membrane is seen to be indented. This indentation has been noticed to be due to the agglomeration of the droplets secreted by the epithelial cells on the surface of the membrane.

These observations indicate that the peritrophic membrane of female *Culicoides nubeculosus* originates as a secretion of the cells of the stomach, which condenses around the ingested blood. Also, that it is composed of successive layers or lamellae.

In flies fixed 24 hr. after the meal, the posterior end of the peritrophic membrane—above the mesenteric sphincter—is seen perforated in the centre, to allow the faecal matter to pass down to the hind-gut through the relaxed sphincter. The membrane by now is mainly brown in colour (light to deep) but faint pink portions may also be seen. Myriads of dark-brown granules and rods—haematin or other derivatives of the blood pigment—are to be seen immersed in its substance (this may be an artifact). One or more layers of the peritrophic membrane may show partial disintegration, but as a capsule enveloping the blood mass the membrane remains intact; it is only at its posterior end that the membrane becomes wholly perforated.

Forty-eight hours after the full blood meal, the blood is practically digested, and the haematin residue makes the identification of the peritrophic membrane very difficult. In some places, however, it can be traced.

Seventy-two hours after the full meal, the stomach becomes empty and crumpled, and the peritrophic membrane has been discharged with the faeces. In one female dissected after laying her eggs, during the fourth day after a full blood meal, the peritrophic membrane was seen gripped by the mesenteric sphincter and projecting into the hind-gut, as is recorded in some females of *Simulium damnosum* by Lewis (1953).

In females which took an incomplete blood meal, an evident peritrophic

membrane was seen in specimens fixed 5 hr. after the meal. It is composed of one layer which is of considerable thickness, a feature which was also observed in *S. damnosum* by Lewis (1953).

It was found difficult to get a reaction for chitin from this membrane. Sections stained with Bethe's stain gave a negative result. Prof. V. B. Wigglesworth (personal communication) kindly informed me that Bethe's stain is of almost no value as a test for chitin, and that the only satisfactory test is the chitosan test. Guts of flies 1–2 days after a full meal were used; these are less distended with blood and can be dissected out without the least injury to them. The chitosan test also gave a negative result; it should be noted, however, that the chitosan test gave also a negative result with the peritrophic membrane of the larva of *Drosophila*, which is known to be chitinous. The membrane, however, can be isolated by macerating the gut in lactic acid in an oven at 48° C. for a few hours. It was left in the lactic acid in the oven for several days without any change. It appears as a very thin and transparent membranous capsule sprinkled with haematin granules. It stains well with acid fuchsin.

A peritrophic membrane has been known to exist in several blood-sucking Diptera. In *Anopheles maculipennis* (Yaguzhinskaya, 1940), a chitinous peritrophic membrane is formed in the mid-gut each time the latter is filled with blood and on the completion of digestion it is evacuated with the excreta. It envelops the blood as a thin capsule, and in the early stages of digestion it is frequently open at the posterior end, and when the digestion is completed the membrane closes up. Apparently, a new membrane can be added to, or formed over the old one, which partly degenerates in the process of digestion. A peritrophic membrane forms in *Anopheles culicifacies*, *A. stephensi*, *A. subpictus* and *Culex fatigans* when the gut is distended with blood (Pal, 1943). Adler & Theodor (1926) state that in *Phlebotomus papatasii* (and also in *P. minutus* and *P. perniciosus*) a very definite peritrophic membrane is produced. It is a thin white amorphous structure which contains the mass of red cells, and may be likened to a sealed tube closed anteriorly and posteriorly; anteriorly it extends into the 'cardia' and posteriorly into the hind-gut. Dolmatova (1942) states that a peritrophic membrane similar to that of *Anopheles maculipennis* forms round the ingested blood in *Phlebotomus papatasii*. In *Simulium damnosum* and *S. griseicolla* (Lewis, 1950), a peritrophic membrane envelops the ingested blood. In *S. damnosum* (Lewis, 1953), the epithelium of the distended part of the mid-gut (i.e. stomach) secretes the peritrophic membrane which encloses the ingested blood. This membrane is chitinous, tough and inelastic, and is laid down in a series of laminae which probably continue to be added to for a short time. It usually remains complete for over 24 hr. and part of it is seen when the blood has disappeared. A peritrophic membrane also forms in tabanids (Olsufev, after Yaguzhinskaya, 1940). In *Glossina* (Wigglesworth, 1929), the peritrophic membrane is secreted by a group of specialized cells at the anterior limit of the mid-gut, and its secretion is enhanced by a blood meal. In *Stomoxys calcitrans* and *Haematobia stimulans* (Kuzina, 1942), a peritrophic membrane is present in the females, whether they had fed or not; it forms 3–4hr. after emergence, and remains after the blood has all been digested.

There is much similarity between the peritrophic membranes of *Culicoides nubeculosus*, *Anopheles maculipennis* (Yaguzhinskaya, 1940) and *Simulium damnosum* (Lewis, 1953). They are secreted by the epithelium of the stomach only after a blood meal, are composed of several laminae, and rupture posteriorly some time after the meal. It is evident that the peritrophic membrane of these insects is different from the two types reviewed in text-books (e.g. Wigglesworth, 1950, p. 313; Day & Waterhouse, 1953, p. 284): the first type is made up of concentric lamellae separated from the surface of the cells throughout the mid-gut (not agreed upon universally), and the second type is a single uniform layer secreted by a group of cells at the anterior limit of the mid-gut. This difference may be the reason why Day & Waterhouse (1953, p. 288) state that 'there is some doubt whether these are true membranes'. But what is a true peritrophic membrane? Snodgrass (1935, p. 378) identifies the peritrophic membrane as 'a cylindrical membranous envelope surrounding the food in the ventriculus, and sometimes extending into the proctodaeum; generated from the ventricular epithelium, either from all or a part of the length of the latter or from a ring of specialized cells at the anterior end'. This generalized identification applies to the membrane which envelops the ingested blood in these nematoceran blood-sucking flies. Waterhouse (1953), however, found that the membrane of such insects as well as those of a variety of insects hitherto known to lack a peritrophic membrane, are real peritrophic membranes. He repudiates what he says is a rather widely-held belief that the endodermal mid-gut is unable to produce a chitinous membrane. He also raises two interesting points: (1) the generalization that the fluid-feeding forms lack a peritrophic membrane must be modified; and (2) the embryonic origin of the cells at the anterior end of the mid-gut (said by him to have been reported as being ectodermal in origin) which secrete the peritrophic membrane of the second type now requires re-examination.

It is generally agreed upon that the peritrophic membrane protects the cells of the mid-gut from damage by hard or sharp particles of food. Also, a number of dye-feeding experiments suggest that the peritrophic membrane acts as an ultra-filter with a maximum pore size varying somewhat from insect to insect (Day & Waterhouse, 1953, p. 306). The first view certainly does not apply to blood-sucking insects. Yaguzhinskaya (1940) suggests that in *Anopheles maculipennis*, it may have a bearing on the penetration of parasites into the wall of the mid-gut, and that the transmission of causal agents of disease by blood-sucking insects may depend partly on the character of the peritrophic membrane. Lewis (1953) states that the peritrophic membrane of *Simulium damnosum* plays an important part in limiting the number of microfilariae that survive to reach the sausage stage.

THE HIND-GUT

The hind-gut is composed of two regions: an anterior simple tube which shows no differentiation into parts—the small intestine, and a posterior rectum (*s.int.* and *rec.*, Fig. 1).

The small intestine is a narrow tube of moderate length which forms one or two

loops in the abdominal cavity. The anterior portion of the small intestine is capable of much distension during the excretory activity of the mid-gut and the Malpighian tubules. The wall of the small intestine (Fig. 11) consists of a single layer of cubical cells (*epi.c.*) which rests on a basement membrane (*b.m.*), followed by a sparsely arranged double layer of inner circular (*c.m.*) and an outer longitudinal muscles (*l.m.*); the inner end of the cells is covered with a thin cuticular intima (*cut.int.*). The epithelial cells are fairly large—their long axes being parallel to the basement membrane, and the intercellular walls absent. The cytoplasm does not stain well with haematoxylin. The nucleus (*n.*) is conspicuous and has an open arrangement of chromatin. The wall of the small intestine is thrown into small folds; but when the anterior portion of the small intestine is distended with excrement, the folds disappear and the epithelium flattens to a degree proportional to the degree of distension, and the nuclei become elongated and compressed.

The opening from the small intestine into the rectum is guarded by a simple valve—the rectal valve (*rec.v.*, Figs. 12, 13). This valve resembles the oesophageal valve in that it is formed by a slight protrusion of the hinder portion of the small intestine into the lumen of the anterior end of the rectum, and its reflexion forwards to join the rectal wall (Fig. 13); the circular muscles of this region are exaggerated to form the sphincter muscles (*sph.m.*, Figs. 12, 13). It has been observed that the contents of the small intestine do not pass directly into the rectum. These contents keep on moving to and fro in the lumen of the small intestine for some time, as a result of the peristaltic and antiperistaltic movements of the small intestine, before the rectal valve relaxes and they are allowed into the rectum; Graham-Smith (1934) described a somewhat similar phenomenon in *Calliphora erythrocephala*. This observation suggests that, apart from obviating any reflux, the rectal valve in female *C. nubeculosus* for some reason (probably the absorption of some nutrient by the cells of the small intestine) prevents the rapid and untimely entry of the excrement into the rectum.

The rectum is dilated anteriorly into a capacious sac—the rectal sac, and narrows posteriorly to form a short straight tube—the rectum proper—which proceeds direct to the anus. The rectal sac (*rec.s.*, Fig. 13) is a capacious oval chamber which contains two rectal papillae (*rec.p.*), projecting into the lumen, and usually visible through the membranous wall of the rectal sac.

The rectum is thin-walled (Figs. 13, 14), and the epithelium (*epi.c.*) is greatly reduced except where it forms the rectal papillae. A cuticular intima is very difficult to detect except around the rectal papillae. The muscular coat of the rectum is well developed and its arrangement is the reverse of that of the small intestine, i.e. an inner longitudinal (*l.m.*) and an outer circular (*c.m.*) layer of muscles. In transverse sections of the rectal sac (Fig. 14), the circular muscles are seen to be composed of several crescent-shaped bands with obvious striation. The rectal papillae (*rec.p.*, Fig. 13) are composed of large cells with very big nuclei. Each papilla is covered with a thin cuticular intima (*cut.int.*), and its lumen contains a tracheal branch.

Posteriorly, the rectum ends in the anus in the last abdominal segment. The anus is furnished with a strong sphincter muscle.

In *Simulium nigroparvum* (Cox, 1938), the hind-gut is divided into the distal

intestine, the rectal pouch and the rectum. The epithelium of the distal intestine consists of flattened cells, which are raised into folds in the posterior part. Covering the epithelium is a layer of circular muscles which forms a continuous layer at the posterior end. The intima is very thin. The walls of the rectal pouch are extremely thin and the epithelium is greatly reduced except where it forms the rectal papillae. Both muscle layers are very thin. In the rectum, both muscle layers as well as the epithelium are well developed and the cuticular intima can be easily detected. In *Phlebotomus papatasii* (Adler & Theodor, 1926), the wall of the hind-gut consists of two layers of muscles, one longitudinal and external and the other oblique and internal. The lumen is lined by a single layer of cubical epithelium, the inner aspect of which is covered with an exceedingly thin glistening chitinous layer. In the mosquito (Nuttall & Shipley, 1903), the hind-gut is divided into the ileum, the colon and the rectum, and ends with the anus. The ileum is very short and transparent, and may be somewhat dilated near the mid-gut; it is lined with flattened epithelium covered with a cuticular intima. The colon succeeds the ileum, without there being any line of demarcation; it is lined by a single layer of cubical cells, and the muscular coat is well developed, showing a well-marked fenestration or crossing of fibres. The rectum forms a spacious oval chamber into which the colon suddenly opens; it is lined with flattened epithelium. In *Tabanus* (Cragg, 1920), the hind-gut may be divided into an ileum, which forms a single loop in the posterior part of the abdomen, a colon, of slightly greater diameter running straight backwards to open into the rectum. In the tsetse flies (Lester & Lloyd, 1928), the hind-gut is divided into three portions. The first portion or 'proto-rectum' is a narrow powerful tube, the pores of the Malpighian tubules entering it at a small dilatation immediately behind the mesenteric sphincter. At its hinder end is a simple valve formed by a reflexion of the gut wall inwards and backwards to form a nipple-like projection, which protrudes into the next region, the 'meso-rectum'. This region is formed by a sausage-shaped distensible tube, which communicates with the globular 'metarectum' by a small pore on the antero-dorsal wall of the latter.

The rectal papillae are of almost the same structure in all adult Diptera. In the blood-sucking flies they differ in number: in *Culicoides nubeculosus* and *Phlebotomus papatasii* (Adler & Theodor, 1926) they are two; in *Simulium* spp. (Smart, 1935; Cox, 1938), mosquitoes (Christophers, 1901; Nuttall & Shipley, 1903) and *Tabanus* (Cragg, 1920) they are six; in the tsetse flies (Lester & Lloyd, 1928) they are four. According to Engel (1924), the usual number of rectal papillae in the Diptera is four or six, the larger number being confined to the Orthorrhapha; in *Culex pipiens* there are four in the male and six in the female.

Except in the tsetse flies, the rectal valve was not mentioned by any of the afore-cited authors, and it is very likely that it was overlooked. Among the non-blood-sucking Diptera in which a rectal valve was described are *Lucilia sericata* (Wigglesworth, 1932) and *Calliphora erythrocephala* (Graham-Smith, 1934).

THE MALPIGHIAN TUBULES

At the point of junction of the mid- and the hind-gut two Malpighian tubules (Figs 1, 9; *Mal.t.*) of a white or yellowish white colour arise, one on each side of the gut. They are cylindrical, of considerable length, and of practically uniform diameter throughout. They take a convoluted course in the abdominal cavity, extending forwards to the anterior end of the abdomen then recurve and extend backwards to lie beside the rectum. They are bound to the gut with numerous tracheal branches.

At the entrance of the tubes into the gut (Fig. 9), they are seen to be lined for a short distance by relatively small low cubical cells (*a.c.*) which stain blue with haematoxylin. These cells are continuous with and are similar to the cells of the mesenteric sphincter. Very large cells (*p.c.*) soon appear. These cells contain many granules and droplets which conspicuously increase both in number and size after a meal of blood. The nucleus (*n.*) is very large and oval in shape. The inner border is markedly striated (*st.b.*). They are arranged alternately in a double row and surround a central lumen. The alternate arrangement of the cells gives the tube a wavy appearance; as a result of this appearance only one nucleus at most is cut in transverse sections. The lumen of the tubule is somewhat flattened, but during excretory activity it dilates and contains many excretory granules and spheres. The cells rest on a basement membrane. The tubules are richly supplied with tracheae and tracheal end-cells.

The Malpighian tubules of *Culicoides nubeculosus* were found to give attachment to muscular branches of different sources. At the anterior region of the abdomen (Fig. 10) a branch of the alary muscles of the pericardial septum ($m._1$) is seen attached to them. At their basal end (Fig. 9), the tubules were found to receive longitudinal muscle fibres from the posterior end of the mid-gut ($m._2$). At the same place, the tubules receive a branch of the longitudinal muscle fibres of the small intestine ($m._3$) (this may be a small tracheal branch).

Eastham (1924) found that the Malpighian tubules of *Drosophila funebris* give attachment to a branch of the alary muscles of the pericardial septum, and also to circular and longitudinal muscle fibres continuous with the muscles of the mid-gut. In *Rhodnius prolixus*, Wigglesworth (1931*b*) found only a few muscle fibres running out from the mid-gut over the ampullae to the lower end of the Malpighian tubules.

Culicoides nubeculosus has two Malpighian tubules, and that is considered an exceptional number. The usual number in Diptera is four (Snodgrass, 1935, p. 380). However, according to Saunders (1924), it appears that the heleids (larvae at least) are divided fairly evenly in possessing two or three Malpighian tubules, but *Forcipomyia* is the only genus in which both conditions occur. He states that the possession of three is unique among insects; Berlese quotes *Culex* and *Psychoda* with five as the only known examples of 'oligonephrous' insects with odd number of Malpighian tubules, to which may be added *Anopheles*, *Ptychoptera* and the Blepharoceridae, all with the same number. In these the odd one is dorsal, but in the heleids it is ventral.

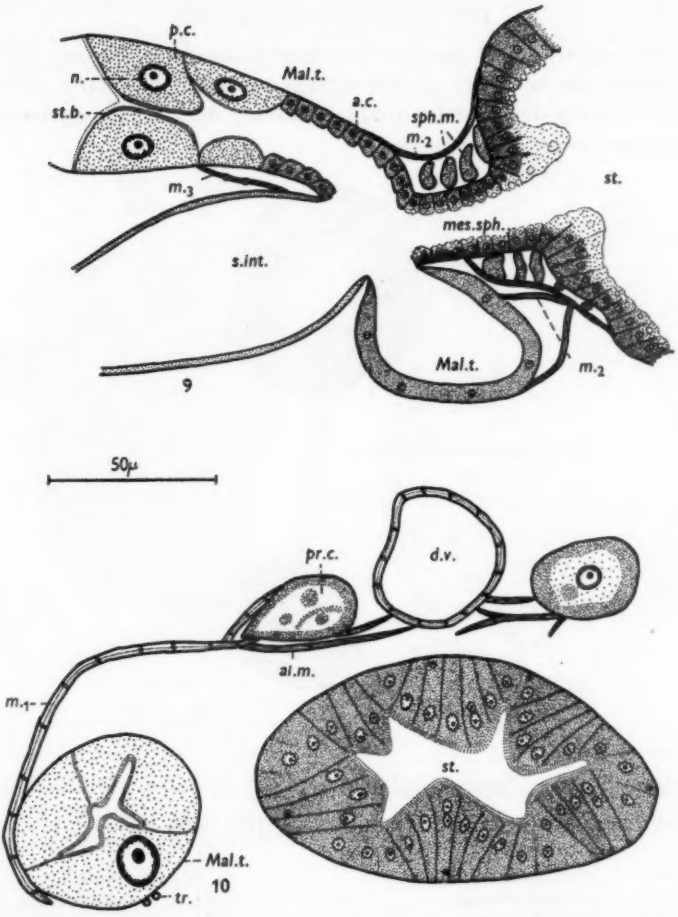


Fig. 9. Longitudinal section through the base of the stomach and the anterior part of the small intestine—showing the structure of the mesenteric sphincter and the Malpighian tubules.

Fig. 10. Transverse section through the anterior end of the abdomen—showing a branch of the alary muscles attached to a Malpighian tubule.

a.c.	epithelial cells at the beginning of the Malpighian tubule	*mes.sph.*	mesenteric sphincter
al.m.	alary muscle	*n.*	nucleus
d.v.	dorsal vessel	*p.c.*	large cells characteristic of the Malpighian tubules
m_1	a branch of the alary muscles	*pr.c.*	pericardial cell
m_2	branches of the longitudinal muscle fibres of the mid-gut	*s.int.*	small intestine
m_3	a branch of the longitudinal muscle fibres of the small intestine	*st.*	stomach
		st.b.	striated border
		sph.m.	sphincter muscle
Mal.t.	Malpighian tubule	*tr.*	trachea.

THE SALIVARY APPARATUS

The salivary apparatus of female *Culicoides nubeculosus* consists of a pair of salivary glands, six accessory glands arranged around the mouth of each gland proper, two salivary ducts, and a common salivary duct, a salivary pump and the hypopharyngeal salivary duct.

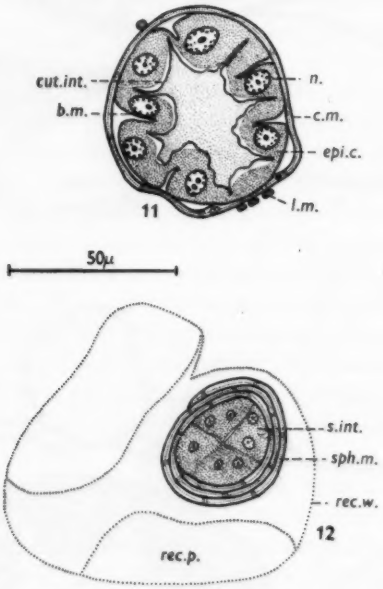


Fig. 11. Transverse section through the small intestine.

Fig. 12. Transverse section through the rectal valve (the rectal wall and rectal papillae in dots).

b.m.	basement membrane	*n.*	nucleus
c.m.	circular muscle	*s.int.*	small intestine
cut.int.	cuticular intima	*sph.m.*	sphincter muscle
epi.c.	epithelial cells	*rec.p.*	rectal papilla
l.m.	longitudinal muscle	*rec.w.*	rectal wall.

The salivary glands

The two salivary glands (*sl.g.*) lie in the lower anterior part of the thorax, ventral to the huge thoracic muscles at both sides of the fore-gut and a little below the anterior segment of the mid-gut. Each gland (Fig. 15, *sl.g.*) is a tube which dilates gradually from before backwards, and bends upon itself in the form of a U. It is not uncommon, however, for one or both glands to lie straight and extend backwards into the anterior portion of the abdomen. In one case only, one of the two glands (with its corresponding accessory glands of which one only could be seen) was seen to extend forwards into the head-capsule; the salivary duct of this gland was obviously shorter than that of the other gland. This seems to be an abnormal case.

The salivary gland consists of a single layer of cubical cells (*epi.c.*, Figs. 17, 18) surrounding a central lumen which contains a granular eosinophil secretion (*sec.*). The intercellular walls are evident, and the cells rest on an obvious basement membrane (*b.m.*). The nucleus (*n.*) is large and conspicuous, oval or round in shape,

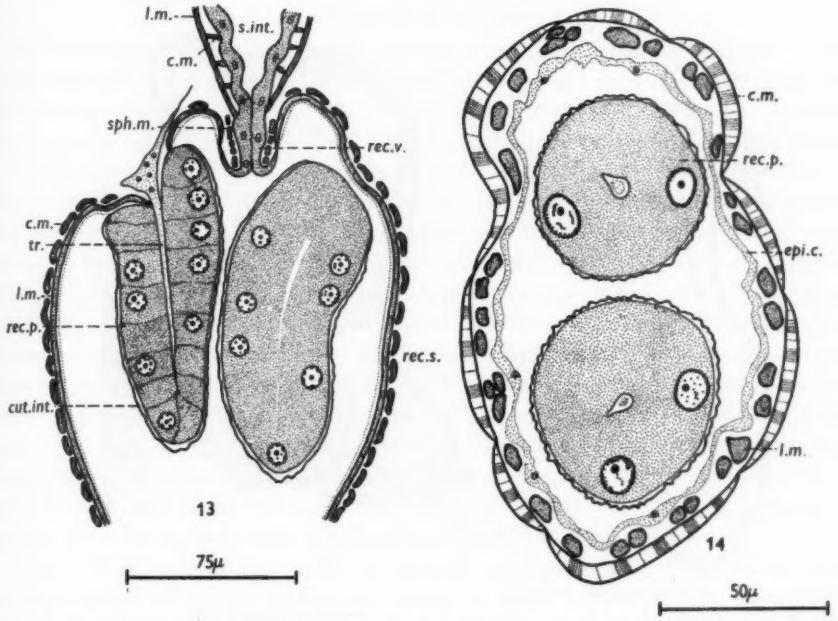


Fig. 13. Horizontal section through the posterior end of the small intestine, and the anterior part of the rectal sac.

Fig. 14. Transverse section through the rectal sac.

c.m.	circular muscle	*rec.s.*	rectal sac
cut.int.	cuticular intima	*rec.v.*	rectal valve
epi.c.	epithelial cells	*s.int.*	small intestine
l.m.	longitudinal muscle	*sph.m.*	sphincter muscle
rec.p.	rectal papilla	*tr.*	trachea

and is centrally situated. The cytoplasm is granular, stains blue with haematoxylin, and no vacuoles can be detected in it. The height of the secretory cells differs according to the degree of distension of the gland with secretion. When the salivary gland is highly distended, the cytoplasm thins out except around the nucleus which never becomes compressed.

In different sections of the lower dilated part of the salivary gland, cytoplasmic strands (*cyt.s.*, Fig. 17), which stain blue are to be seen protruding into the lumen. This is not the case with the cells of the anterior portion of the gland (Fig. 18). The actual relation of these strands was difficult to ascertain. In the first sections of this part of the gland, these strands are seen to come together in a network with the granular eosinophil secretion filling its meshes, except in one or two positions

where complete polygonal cells are seen. In sections of flies killed after a full meal of blood, these strands are also seen. These observations may suggest that these strands are the remains of cells removed in previous sections. But, at any rate, it is also probable that the free end of the cells forms a pocket in which the secretion accumulates, and when the pocket becomes fully distended it eventually bursts

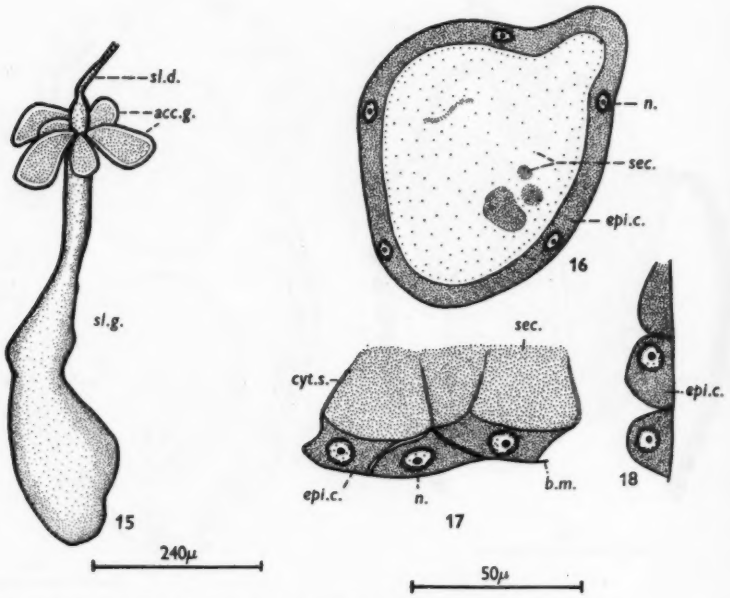


Fig. 15. Diagram of a salivary gland and its accessory glands.

Fig. 16. Horizontal section through an accessory gland.

Fig. 17. Transverse section through the posterior end of the salivary gland.

Fig. 18. Longitudinal section through the anterior end of the salivary gland.

acc.g.	accessory gland	*n.*	nucleus
b.m.	basement membrane	*sec.*	secretion in the lumen of the gland
cyt.s.	cytoplasmic strands	*sl.d.*	salivary duct
epi.c.	epithelial cells	*sl.g.*	salivary gland

open to discharge the secretion into the lumen, leaving its sides as such protrusions. In the anterior part of the gland, though the lumen is also to be seen more or less full of the granular eosinophil secretion, the cells were sometimes seen discharging small cytoplasmic droplets which stain blue.

Directly after a full blood meal, the salivary glands contain only a trace or, sometimes, a very little amount of secretion. This is different from what occurs in the salivary glands of mosquitoes which, according to Christophers (1901), for the most part are still quite full of secretion after feeding. In a series of females sectioned after 1, 5 and 24 hr. after a full blood meal, the salivary glands contained increasing amounts of secretion. The method of secretion is not known.

After a small meal of blood the salivary glands contain considerable amounts of secretion; more or less inversely proportional to the amount of the ingested blood. This observation suggests that the saliva is not forced into the wound at once, but is discharged throughout the period of feeding.

The accessory glands

The accessory glands (*acc.g.*, Fig. 15) are six small saccular structures, three small and oval and three rather big and rectangular, arranged alternately in a rosette around the mouth of each of the salivary glands proper, each sac opening into it by means of a very tiny duct. They lie partly in the thorax and partly in the neck; the dissection of the salivary glands with the whole set of accessory glands is a very delicate job, and much concentration is required otherwise some of the sacs will be torn out. Each sac (Fig. 16) consists of a single layer of low or flat cells (*epi.c.*) which rests on a basement membrane, and surrounds a relatively wide lumen. The intercellular walls are absent. The nucleus (*n.*) is small and oval in shape. The cytoplasm is granular and stains blue with haematoxylin. In many of these sacs the lumen appears to be empty or contains a little quantity of a homogeneous secretion (*sec.*), which very faintly stains with eosin. In some others, traces of secretion similar to that found in the salivary glands proper, a little amount of a granular secretion which stains blue, and a few glistening, unstained, small droplets may be seen in the lumen. In only one specimen, a fly fixed 2 days after a full blood meal, two sacs only were found full of a granular eosinophil secretion similar to that seen in the salivary glands proper.

Patton & Evans (1929, p. 140) mentioned and figured only four sacs to each salivary gland of female *Culicoides varius*(?). They suggested that these sacs probably serve as temporary reservoirs for the secretion of the salivary glands. In *C. nubeculosus*, these sacs have been shown to be active secretory glands, the secretion of which is of a different nature from that of the salivary glands proper, and in rare instances some of them may serve as reservoirs for the secretion of the salivary glands proper. This case is somewhat similar to what has been described in some of the Hemiptera-Heteroptera, i.e. *Notonecta*. According to Baptist (1941), the accessory gland of *Notonecta* is vesicular, the glandular epithelium is thin and flattened with small round nuclei, and it secretes a watery fluid but may serve as a reservoir of the principal gland under special circumstances.

The presence of accessory glands in *Culicoides nubeculosus* is very interesting as, so far as I am aware, these have not been described in any other blood-sucking fly.

The salivary ducts

At the point of junction between the salivary gland and its accessory glands emerges the salivary duct which is wide to begin with but soon narrows down (*sl.d.*, Fig. 15). The salivary duct of each side passes through the neck into the head-capsule, and, a little distance in front of the occipital foramen and beneath and in contact with the suboesophageal ganglion, the two ducts converge to the middle line where they unite to form the common salivary duct. The common salivary

duct passes forwards till it opens into the salivary pump underneath the cibarial pump.

The salivary ducts and the common salivary duct are annulated tubes with chitinous walls covered with a thin layer of flat epithelium.

The salivary pump

The salivary pump of *C. nubeculosus* resembles that of *C. pulicaris* (Jobling, 1928) and *C. impunctatus* (Gad, 1951). According to Snodgrass (1943), the salivary pump is present in all the Diptera.

There are, however, some specific differences between the salivary glands in the blood-sucking flies. In *Simulium* (Smart, 1935; Cox, 1938), the two salivary glands are tubular and U-shaped, each with a round reservoir from which passes the salivary duct. In *Phlebotomus papatasii* (Adler & Theodor, 1926), they are almost spherical. In mosquitoes (Christophers, 1901; Nuttall & Shipley, 1903), each salivary gland is composed of three tubular acini lying one above the other in the long axis of the body. Each acinus has an intra-glandular or intra-acinar duct; shortly after leaving the acinus the three intra-acinar ducts unite to form a single duct. A common abnormality is a small accessory acinus near the proximal end of an acinus (Christophers, 1901); Roy & Mayne (1931) observed supernumerary acini in the salivary glands of several anopheline mosquitoes. The middle acinus differs from both the two lateral ones. Christophers mentions that the middle acinus is of the clear or colloid-like type and, in preparations, its secretion is clear and homogeneous, and stains readily, sometimes quite deeply, with haematin; the other two acini are of the granular type, and their secretion is granular and stains faintly with haematin. Nuttall & Shipley, on the other hand, state that the secretion of the lateral acini does not stain with eosin as does that of the middle one.

A NOTE ON TUMOURS IN THE ALIMENTARY TRACT, AND ABNORMAL FEATURES IN THE MALPIGHIAN TUBULES

In the course of this study of the alimentary tract of female *Culicoides nubeculosus*, tumours were observed in one specimen only. These (*tu.*, Fig. 2) were several protruding, well defined, saccular swellings of different sizes situated at the base of the stomach (*st.*). Two of them had a triangular dark-brown area, and one had a small round spot of the same colour. The cause and pathogenicity of these tumours are not known. It would be interesting to know if these tumours occur in the developing stages and, if they do occur, to what extent they affect these stages.

Very few insects are known to develop tumours. In *Drosophila melanogaster* (Russell, 1940, 1942) both non-genetic and multiple genetic factors influence the production of tumours. None of these tumours is malignant and all of them may be transplated successfully without hampering the development of the host. It is suggested that pupation may restrict these abnormal growths and prevent their becoming malignant.

In the cockroach *Leucophaea maderae* (Scharrer, 1945, 1948), severance of the

recurrent nerve causes the development of tumours in organs supplied by this nerve. The sites of experimentally induced neoplastic growths are the fore-gut, the anterior mid-gut, the salivary glands, and the salivary reservoirs. These tumours are well-defined conspicuous tissue masses, which in advanced stage can be easily noticed with the naked eye. Features indicating the malignant character of these tumours are their tendency to invasive growth and their transplantability. A few specimens not operated upon showed changes in the alimentary canal which, although of milder degree, were comparable to the tumours developing after the section of the recurrent nerve. According to Scharrer (1945), a brain tumour in an ant was described by Brun, and allatectomy of young nymphs of the orthopteran *Dixippus* was shown by Pflugfelder to be followed by tissue growth and degeneration. The European spruce sawfly *Gilpinia hercyniae* (Bird, 1949) develops non-malignant tumours after infection with the virus causing a 'virus polyhedral disease'. In the diseased larva the virus affects only the digestive cells of the mid-gut epithelium. Most of the proliferations project into the body cavity, but infiltration into the cytoplasm of the digestive cells does occur. Large tumours are formed only when the larva becomes infected just prior to the last larval moult. The tumours rapidly mature during an early stage of pupation. In the adult some of the tumours may be pushed out into the body cavity during pupation, where they remain as non-living, hard, dark-brown pellets throughout the life of the adult.

Another abnormality in the same specimen was seen in the Malpighian tubules (*Mal.t.*, Fig. 2). In normal females the two Malpighian tubules are cylindrical and of a whitish or yellowish colour. They extend forwards to the anterior end of the abdomen, then recurve and extend backwards to lie besides the rectum (Fig. 1). In this specimen they were of a deep orange colour. The base of each tubule was swollen and ampulla-shaped (*amp.*, Fig. 2) followed by a small narrow area after which the tube was flattened for a considerable length before it took its usual cylindrical shape. Each tube was coiled in a mass, intermingled with many tracheae and tracheal branches, and lay beside the hind-gut. It is not known if these abnormal features in the Malpighian tubules are related to the presence of the tumours in the base of the stomach.

SUMMARY

The alimentary tract of female *Culicoides nubeculosus* is simple. There is much similarity between it and that of other nematoceran and brachyceran blood-sucking flies.

Among the interesting features observed are the following:

1. The mid-gut is composed of two portions, a tubular anterior segment and a dilated stomach, which show structural differences.

2. The peritrophic membrane, which is absent in the unfed female, forms around the ingested blood. Its substance is secreted by the epithelium of the stomach.

3. A simple rectal valve guards the opening of the small intestine into the rectum.

4. The Malpighian tubules are two in number, and they give attachment to muscular branches from different sources.

5. There are six accessory glands arranged in a rosette around the mouth of each salivary gland proper. Some of them may sometimes function as reservoirs for the secretion of the salivary glands proper.

In one specimen, tumours at the base of the stomach and abnormal features in the Malpighian tubules were observed.

This work was carried out in the Department of Zoology, University of Glasgow. I wish to record my grateful thanks to Prof. C. M. Yonge, O.B.E., F.R.S., for the facilities put at my disposal in his department. I am also indebted to Mr J. A. Downes for presenting me with the culture of *C. nubeculosus*, for suggesting the subject, and for directing the work in the early stages. I have pleasure in thanking Dr J. W. H. Lawson for his indispensable advice and helpful criticism, and for reading the manuscript.

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PEREZIA SP. (FAM. NOSEMATIDAE) PARASITIC IN THE FAT-BODY OF *GONOCEPHALUM ARENARIUM* (COLEOPTERA: TENEBRIONIDAE)

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(With 15 Figures in the Text)

The microsporidian described in this paper is parasitic in the fat-body of *Gonocephalum arenarium* (Order Coleoptera; Family Tenebrionidae), a root-feeding beetle which is usually found sheltering under stones or fallen leaves and in lawns in South Africa. During the present investigation only adult insects were available for study as the earlier phases of the life history doubtless occur beneath the surface of the soil.

MEROGONY

The extrusion of the filament from the spore and the emergence of the sporoplasm (infective stage) of the microsporidian in question into the tissue of a new host almost certainly takes place in the gut. It must be emphasized, however, that no form of the parasite has at any time been detected within the lumen of the gut or in the gut-cells. If wandering planonts actually occur in the alimentary tract it is likely that they would have been observed, as many hundreds of the insects had been examined. Nevertheless, the sporoplasm must be capable of passing through tissue, since it must reach the fat-body before further development can take place. On penetrating the fat-body the organism commences multiplication by binary fission. The resultant bodies (meronts) each measure some 2·5 μ in diameter and possess an eccentrically situated nucleus. When dry-fixed and stained by Leishman's method the nucleus appears as an almost solid block of chromatin (Fig. 1), but when wet-fixed and stained with haematoxylin the nucleus exhibits a vesicular structure with a relatively large karyosome. The latter appearance of the nucleus is consistent throughout all subsequent resting stages in wet-fixed material (Fig. 6). The cytoplasm of the meronts stains intensely and evenly with Leishman's stain. Complete separation usually follows each division but a few multinucleate plasmodia are usually to be found. Binucleate forms are very numerous (Fig. 2), while elongate quadrinucleate bodies resulting from a precocious nuclear division of the daughter-nuclei of two separating cells are very common (Fig. 3). Multiplication by schizogony does not occur.

In the earliest stage observed the meronts are found lying in isolated clusters which are more or less evenly dispersed throughout the portion of the fat-body involved. These clusters may measure as much as 40 μ in diameter. The presence of an enveloping membrane is problematic. It is within these groups that the organism passes through all stages of development. There is no indication of the

secretion of a histolytic enzyme and the parasites do not, therefore, lie in vacuoles in the adipose tissue, nor does the group appear to be associated with any particular host-cell. It appears, rather, to lie free in the cytoplasm of a number of cells. Very soon, and while the infection is confined to a small portion of the fat-body, the meronts increase in size until they measure about 4·2 μ in diameter (dry-fixed) and become pansporoblasts (Fig. 4).

SPOROGONY

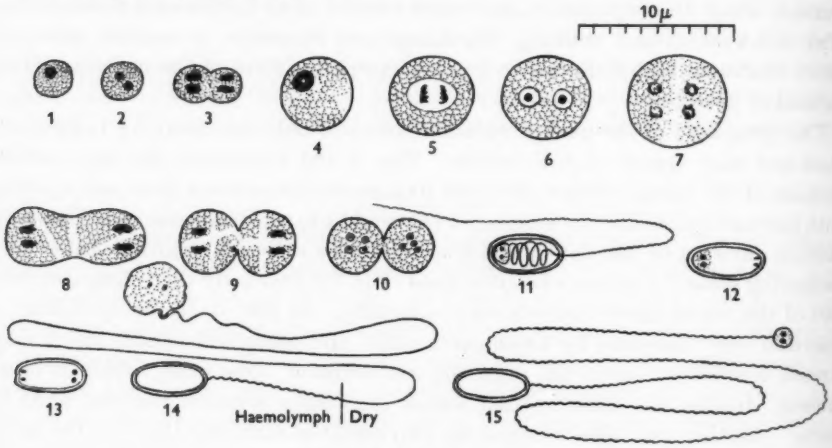
The development of the pansporoblast and the sporoblast can best be followed in dry-fixed material, as it has been found much easier to identify the different stages through which the organism passes when treated after Leishman's method than after wet-fixation and staining. Shrinkage and distortion is marked after the latter treatment and little can be learned except the form of the nucleus and its method of division.

The cytoplasm of the pansporoblast is colorized only delicately by Leishman's stain and may appear slightly patchy. Figs. 5 and 6 illustrate the first nuclear division of the pansporoblast after wet-fixation in Schaudinn's fluid and staining with haematoxylin. This division is not followed by cytoplasmic cleavage. A simultaneous division of the two nuclei then produces a round quadrinucleate body measuring some 7 μ across when dry-fixed (Fig. 7). This body then elongates, and two of the nuclei move towards each extremity. At this stage the cytoplasm is colorized very unevenly by Leishman's stain, and unstained bands, which may stretch completely across the organism are common. One band, which is often present, divides the pansporoblast across the centre between the two pairs of nuclei. Another may pass between the two nuclei of each pair (Fig. 8). The body then constricts across the middle and two sporoblasts are formed (Fig. 9). Each sporoblast is at first more or less round and contains two nuclei, the cytoplasm stains very delicately, and unstained bands are constantly present. They then become subspherical and later oval, while the internal contents become masked by the development of the cyst-wall which appears to be formed very quickly. When wet-fixed and stained with haematoxylin, the presence of four minute nuclei can be detected before the developing cyst-wall renders the study of the internal structure impossible (Fig. 10). Separation of the two developing sporocysts takes place before the cyst-wall is completely formed so that mature spores do not adhere together in pairs.

It has not been possible to study the development of the coiled filament, nor has this organelle been seen either in the fresh state or in stained material before its extrusion from the sporocyst. After extrusion has taken place dyes are apparently able to penetrate the interior of the cyst which is then generally found to be empty, but if extrusion has not been complete a portion of the filament can be observed coiled around the interior of the cyst, while the sporoplasm lies at the posterior end (Fig. 11). A definite polar capsule has not been observed.

Except as described above, the filament has never been seen *in situ*, but, in a few well-staining spores, various nuclei are rendered visible by Leishman's stain.

The picture observed is not always the same, as naturally this would be affected by the orientation of the spore relative to the observer and also by its stage of development, but generally the cyst shows two nuclei near the posterior end, while two smaller chromatic bodies lie at the anterior tip. The posterior nuclei, which are almost certainly those of the sporoplasm, are enveloped in a mass of cytoplasm (Fig. 12). Hall (1952*a*), in a paper dealing with *Perezia pyraustae*, describes the fusion of two sporoplasm nuclei into one within the spore before the extrusion of the filament. It is possible, however, that the sporoplasm may have appeared to be either uninucleate or binucleate according to the orientation of the sporocyst relative to the observer, when obviously it must occasionally happen that the two nuclei are optically superimposed.



Figs. 1–15.

Figs. 1–3. Meronts. Fig. 4. Pansporoblast. Figs. 5, 6. First nuclear division of pansporoblast. Fig. 7. Quadrinucleate pansporoblast. Fig. 8. Pansporoblast forming two sporoblasts. Fig. 9. Two sporoblasts. Fig. 10. Two sporoblasts each with four nuclei. Fig. 11. Sporocyst with partly extruded filament. Figs. 12, 13. Sporocysts. Fig. 14. Sporocyst lying in haemolymph, with filament extruded over dry area and ejected sporoplasm. Fig. 15. Sporocyst with filament extruded in haemolymph and with sporoplasm adhering to its tip. *Note.* Figs. 5, 6, 10, 11 and 13 were wet-fixed and stained with haematoxylin; all other figures were dry-fixed and stained by Leishman's method.

Wet-fixation causes distortion of the cyst-wall which often becomes truncated or even depressed at the anterior tip (Fig. 13). The wall of the cyst is equally thick at all points, measuring about 0·25 μ.

FILAMENT EXTRUSION

With the exception of the somewhat crude method of pressing on the cover-glass, no means has been discovered of inducing filament extrusion in the case of spores which have not been dried, but after desiccation for a few hours a certain proportion of them will extrude their filaments in the haemolymph of the host, in normal saline, or even in distilled water. The numbers which react to the haemolymph

of the host are far greater than when saline is used, while a very poor reaction is observed in water. Large numbers of spores have reacted in the haemolymph after being subjected to desiccation for 9 weeks.

The extrusion of the filament is not as vigorous a phenomenon as in most other species. It is not shot out with the usual explosive force, and several seconds are required for it to become fully extended. It is very fine and difficult to observe by bright-ground illumination, and attains a length of about 100 μ. The coils are extremely close and of very small diameter, apparently not deviating from straight by more than two or three times the thickness of the filament. When the filament is completely extruded the sporoplasm appears at its tip. The sporoplasm is a binucleate body measuring about 1·75 μ across; the cytoplasm and the nuclei stain deeply and evenly by Leishman's stain (Fig. 15). It is difficult to obtain stained specimens when the filament has been extruded in normal saline, as the sporoplasm becomes detached and disintegrates, but if the haemolymph of the host is employed to induce extrusion, good stained preparations can be obtained.

It has recently been shown (Gibbs, 1953) that the spores of *Gurleya* sp. extrude their filaments in saline or the haemolymph of the host after a period of desiccation. It was pointed out that, as soon as the filament was sufficiently quiescent for observation, the sporoplasm could be seen adhering to its free end. A method was described of inducing the filament to pass over a semi-dry surface during the course of its extrusion, and thus reduce its rate of movement so greatly that it could definitely be observed that a quantity of protoplasm exuded from its tip at the moment the filament was fully extended. When the protoplasmic droplet was fixed and stained it was found to contain a single nucleus. The sporoplasm, when ejected into a liquid medium, similarly exhibited a single nucleus. From these observations it was inferred that the sporoplasm emerges from the tip of the tube-like filament when its extrusion is completed.

With certain distinctions, virtually the same phenomenon is observed in the present microsporidian. If air-bubbles are induced in the medium in which the spores are mounted, those lying in the liquid near the margin of the bubble will, if suitably orientated, extrude their filaments into the area devoid of liquid. The progress of the filament across the dry area is very slow, although for about the last twentieth of its travel it speeds up considerably, and as it stops abruptly the sporoplasm pours from its tip. In contrast to *Gurleya* sp., in which the filament moves in a markedly even serpentine manner across the dry area, the filament of the present organism travels without undulations, although it may change its direction. During the last part of its travel, however, when as stated it moves considerably faster, the serpentine movement is very obvious. When ejected upon a dry surface the sporoplasm becomes frothy and irregularly shaped, measuring on an average 6 μ across and containing two nuclei (Fig. 14).

In the present organism it is not necessary to resort to extrusion over a semi-dry surface in order to observe that the sporoplasm is ejected from the tip of the filament; extrusion in saline or in the haemolymph takes place so slowly that it can readily be seen, if the tip of the extending organelle is accurately followed and focused, that it carries no appendage until movement ceases, at which moment

a small globule suddenly appears and remains adherent for a short time (Fig. 15), after which it becomes detached. As soon as the sporoplasm appears at the tip of the filament, it is seen that the sporocyst is empty. The detachment of the filaments from the sporocysts has never been observed, even when they were kept in the haemolymph or saline in a sealed chamber for twelve hours.

EFFECT OF PARASITE ON HOST

In sectioned material it is found that the cytoplasmic borders of the adipose tissue cells disappear as the infection progresses, and later their nuclei alone appear to remain scattered among a mass of parasites. In healthy adipose tissue the nuclei measure some 8 μ in diameter and are approximately evenly spaced throughout the fat-body, whereas in heavily infected tissue the nuclei are degenerate. Instead of being spherical they may appear elongate or moon-shaped, while the rounded forms may measure anything from 7 to 30 μ in diameter. They are not evenly spaced throughout the tissue, but often lie in clusters of from three to six nuclei. At a later stage there does not appear to be any fat-body left; all that can be made out are the distorted nuclei lying among a mass of spores. Later even the nuclei may disappear from large portions of the fat-body, and eventually the body-cavity becomes packed with spores, which occupy a space many times that of the healthy fat-body.

Elizabeth Canning (1953) reports a similar replacement of the entire fat-body tissue of *Locusta migratoria* by a mass of spores of *Nosema locustae*, which is definitely pathogenic to its host. In the present case no way has been found of satisfactorily maintaining the insects in captivity, therefore it cannot be stated whether, or not, the life of the insect is shortened by the presence of the parasite. The spores are never found in the dejecta of the insects and must, therefore, be disseminated after the death and disintegration of the host.

DISCUSSION

The passage of the filament across a dry surface, while the sporocyst lies free in a liquid medium, supports the hypothesis that this organelle consists of a coiled hollow tubule which has to be completely everted during extrusion, indeed it is difficult to visualize any other mechanism by which this movement could be affected. Considerable pressure must be developed within the cyst to effect the eversion unless the process is aided by some form of tension on the part of the filament to evert itself, and as extrusion can be induced by pressure with the finger on the cover-slip, it is obvious that no great pressure is involved. The passage of the sporoplasm through the hollow filament is difficult to comprehend but its viscosity is very low, as is indicated by the almost instantaneous manner in which it is gushed from the filament to form a small shapeless pool when ejected on a dry surface. Whether the sporoplasm is actually carried along by the inner fold of the everting tubule, or whether it has to be forced through its entire length when completely extended, is a matter for conjecture. The increase in the rate of movement

during the last part of the extrusion is consistent with the eversion hypothesis, as naturally friction would become reduced as the filament becomes extended.

Almost immediately after filament extrusion has occurred, the sporocyst can be observed to empty itself, both in the case of *Gurleya* sp. and the present *Perezia* sp., and the presence, within it, at this stage, of a sporoplasm which must creep out cannot be seriously considered. Hall (1952*b*) figured the emergence of the sporoplasm from a spore of *Nosema infesta*, but he employed pressure to induce filament extrusion and his interpretation of the figure may have been incorrect. When this artificial method of inducing extrusion is employed, the sporocysts become ruptured, the filaments are found at all stages of extrusion or may be completely torn away, while the protoplasmic contents of the cysts may remain *in situ*, or partially or entirely squeezed out. If, as is commonly supposed, the filament is merely an organelle for the attachment of the spore to tissue suitable for penetration and development of the sporoplasm, that purpose must be frustrated if the filament becomes detached from the spore before the sporoplasm emerges, since the anchorage would be lost. From this point of view it seems strange that it has so frequently been stated that the sporoplasm creeps out of the sporocyst after the extrusion and detachment of the filament. As stated above, the filament has never been observed to become detached.

Since the pansporoblast consistently produces two sporoblasts the organism qualifies for inclusion either in the genus *Glugea* Thélohan or in the genus *Perezia* Léger & Duboscq. As Hall (1952*a*) aptly comments, these two genera are distinguished not on the basis of their own characteristics but upon pathological changes occurring within the host tissue. The present organism does not cause hypertrophy of the host-cells as far as has been observed and does not lie, at any stage, within cytoplasmic vacuoles formed in the host-cell. On this account it is decided to place the described microsporidian provisionally in the genus *Perezia*, but it is not desirable, without further study and comparison, to propose a specific name.

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THE LIFE HISTORY AND DEVELOPMENT OF *TOXOCARA CATI* (SCHRANK 1788) IN THE DOMESTIC CAT

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(With Plate I and 29 Figures in the Text)

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I. INTRODUCTION

(1) *Nomenclature*

Goeze (1782) recognized two ascaridid nematodes in the domestic cat. He differentiated one (p. 79) which possessed 'zwo länglichte, abstehende, durchsichtige, weissgrauliche Blasen, wie Backenbärte' as distinct from the other which he described as 'keine Backenbärtige' (p. 80); he included them both under the group name *Ascaris teres*.* Goeze's figure of the former parasite (pl. I, fig. 5) shows that he had observed the broad-winged ascaris from the cat. Schrank (1788, p. 8), referring to this figure, applied the name *A. cati*, and his name has priority. Schrank mentioned the second species, observed but not figured by Goeze, and left it unnamed, which indicates that his species was not a mixed one. Gmelin (1790, p. 3031) gave each of Goeze's two parasites separate specific names, namely *A. felis* and *A. cati*. The former he described as having greyish-white oblong vesicles on each side of the head, and it clearly corresponds with *A. cati* Schrank. *A. cati* Gmelin was distinguished from *A. felis* Gmelin by the absence of vesicles on the head. Rudolphi (1793, p. 11) listed only *A. felis* Gmelin from the cat; he pointed out that it possessed semi-ovate membranes on the head, which distinguished it from a similar parasite in the dog, which had semi-lanceolate membranes. The latter parasite was first described by Werner (1782) as *Lumbricus canis*, and was renamed *Ascaris werneri* by Rudolphi (1793, p. 11). Zeder (1800, p. 45) included the above worms in his genus *Fusaria*, but introduced a new specific name for the parasite of the cat, which became *F. mystax*. Rudolphi (1802, p. 13), accepting

* *Lumbricus teres* Tyson, 1683, was renamed *Ascaris lumbricoides* by Linnaeus, 1758.

Zeder's specific name but rejecting the genus *Fusaria*, listed *Ascaris mystax* from the cat, and once more renamed the parasite of the dog which he called *A. marginata*.

Rudolphi's rejection of previous specific names for these two parasites in favour of *mystax* and *marginata* was accepted by Diesing (1851) and Cobbold (1864). Leuckart (1876, p. 258) believed that differences in the cervical alae were artificial and due to different methods of fixation; he regarded the ascaridids of both dogs and cats as one species, *Ascaris mystax*, and this was accepted by Neumann (1892), Moniez (1896) and Stossich (1896). Stiles & Hassall (1905, p. 150) created the genus *Toxocara*, designating *Lumbricus canis* Werner, 1782, as type species. Leiper (1907) created two genera of winged ascaridids: *Belascaris*, with *Ascaris mystax* (Zeder, 1800) from the cat as type species; and *Toxascaris*, with *Ascaris leonina* von Linstow, 1902, from the lion as type species. He distinguished the former genus by the ventriculus at the posterior end of the oesophagus, the probular tail of the male, corrugated egg-shell, and other morphological features; but as later pointed out by Railliet & Henry (1911) he was wrong in including Rudolphi's *Ascaris marginata* (=*Toxocara canis*) in the genus *Toxascaris*. Glaue (1909) compared the cervical alae of the ascaridids of the dog and cat; he showed that contrary to the views of Leuckart, two different species were involved. He referred to them as *Ascaris canis* and *A. felis*. Railliet & Henry (1911) first showed that there are, in fact, two narrow-winged ascaridid nematodes occurring in the dog, one belonging to Leiper's *Belascaris*, and the other belonging to *Toxascaris*. Basing their conclusions on Schneider's re-examination of Rudolphi's original specimens of *Ascaris marginata*, they named the first *Belascaris marginata* making *Toxocara canis* a synonym; the second they named *Toxascaris limbata*. It was undoubtedly this latter species which was referred to by Leiper (1907) as *Toxascaris marginata* and by Glaue (1909) as *Ascaris canis*.

Taylor (1924) compared *Toxascaris leonina* from the lion with *T. limbata* from the dog and a similar parasite from the cat; he concluded that they were both identical with *T. leonina*. The priority of *Toxocara* over *Belascaris* was confirmed by Stiles & Brown (1924) and their combination, *Toxocara mystax*, has been in use up to the present time. There is no doubt, however, that the correct name for the parasite is *T. cati* (Schrank, 1788).

It is evident from the foregoing that considerable confusion has existed over the identity of the three ascaridids of dogs and cats. Furthermore, it was not until 1924 that it was conclusively established that the cat may harbour two ascaridid nematodes. Unfortunately they both possess cervical alae, and none of the more precise differentiating characters was known to the older observers. Hence it is often difficult or impossible to determine from the older reports which of these two parasites was actually being mentioned, and it has been difficult to establish a precise list of synonyms for *T. cati*. Nevertheless, it has been possible to revise considerably the list of synonyms given by Stossich (1896), and to remove the following ten of his fourteen synonyms:

(1) *Ascaris canis*; (2) *A. marginata*; (3) *A. microptera*; (4) *A. triquetra*; (5) *A. vulpis* (these are all names of narrow-winged ascaridids of Canidae); (6)

A. brachyoptera Rudolphi, 1819, from *Genetta genetta*, later reported from the coati, *Nasua narica* by Diesing (1851, p. 182), who stated that it has semi-elliptical wings; (7) *Ascaris mustelarum* Rudolphi, 1819, from *Martes* spp. should be regarded as a doubtful species (Sprent, 1952*b*); (8) *Ascaris alienata* Rudolphi, 1819, from *Nasua rufa*; this parasite does not possess cervical alae (Sprent, 1952*b*); (9) *Ascaris macroptera* Diesing, 1857 (p. 13, pl. I, figs. 18–23) from the crocodiles, *Champsa nigra* and *Jacare guacu*; Diesing's description and figures indicate that this was a broad-winged ascaridid resembling *Toxocara cati*, but in view of the wide differences in the hosts, this species should not be regarded as a synonym of *T. cati*; (10) *Ascaris anterospiralis* Molin, 1860, from *Felis concolor*; von Drasche, 1883 (p. 130, plate 10, figs. 14–15) re-examined this species and reported that it had narrow cervical alae.

Ascaris leptoptera Rudolphi, 1809, was not included as a synonym of *Toxocara cati* by Stossich, but was listed as such by Railliet & Henry (1911), and as a partial synonym by Yorke & Maplestone (1926). This is probably erroneous, as it seems likely from Rudolphi's description that he was describing the narrow-winged ascaridid of the lion, as he differentiated and named this species by its long, narrow cervical alae. On the other hand, it is not possible to decide with certainty, either from Rudolphi's own drawings or from Bremser's drawings of Rudolphi's material (Bremser, 1824), that *Ascaris leptoptera* Rudolphi was what is now called *Toxascaris leonina*. The latter was described by von Linstow (1902) from the lion and it was differentiated from *Toxocara cati* by the smooth egg shell and by the gradually tapering cervical alae. Smith, Fox & White (1908) described similar parasites from the lion, but tentatively retained Rudolphi's name *Ascaris leptoptera*. It seems that *A. leptoptera* should be regarded as a nomen confusum, but not as a synonym for *Toxocara cati*.

A revised synonymy is therefore as follows:

Toxocara cati (Schrank, 1788); Brumpt, 1927 (*Précis de Parasitologie*, 4th ed.).

Objective synonyms (based on Goeze's material)

Ascaris teres Goeze, 1782 (in part), not *Lumbricus teres* Tyson, 1683.
A. cati Schrank, 1788, not *Ascaris cati* Gmelin, 1790.
A. felis Gmelin, 1790; Rudolphi, 1793; Glaue, 1909.
Fusaria mystax Zeder, 1800.
Ascaris mystax (Zeder, 1800); Rudolphi, 1802; Leuckart, 1876 (in part); Stossich, 1896 (in part); not *A. mystax* Nelson, 1852.
A. mystax var. *felis* Railliet, 1895 (*Traité de Zoologie Medicale*, 2nd ed. p. 402); Gough 1909.
Belascaris mystax (Zeder, 1800); Leiper, 1907.
B. cati (Schrank, 1788); Brumpt, 1922.
Toxocara mystax (Zeder, 1800) Stiles & Brown, 1924.
T. felis (Goeze) Brumpt, 1936 (*Précis de Parasitologie*, 5th ed., evidently a lapsus for Gmelin).

Subjective synonyms (based on other material)

Ascaris alata Bellingham, 1839, from man. See Cobbold, 1864 (*Entozoa*, pp. 316–29, figs. 68–69). Not *A. alata* Linst., 1883.
A. canis Werner var. *felis* Galli-Vallerio, 1901 (*Bull. Soc. Vaudoise Sci. Nat.* p. 363).
A. circumflexa Molin 1858 (*S.B. Akad. Wiss. Wien*, Bd. 30, p. 145) from *Felis pardus*.

**A. crenulata* Bremser, 1824 (see Railliet & Henry, 1911) from *Felis onca*. Not *Ascaris crenata* Schrank, 1790; not *A. crenata* Rudolphi, 1808.

A. leptoptera var. *crenulata* Diesing, 1851, p. 179, from *Felis concolor* and *F. macroura*.

Toxocara crenulata (Bremser, 1824); Yorke & Maplestone, 1926; Canavan, 1929, from *Lynx canadensis*.

Belascaris crenulata (Bremser, 1824); Railliet & Henry, 1911.

Ascaris felis Glaue, 1910 (*Zool. Anz.* **35**, 748, fig. 1F), from *Felis catus ferus*.

Ascaris globulus von Linstow, 1899 (*Mitt. zool. Samml. Mus. Naturk. Berl.*, v.1., p. 5, plate I, figs. 1–2), from *Felis moormensis*.

Ascaris mystax var. *leonis* Vallillo, 1909 (*Zbl. Bakt.* (1. Orig.), **50**, 461), from *Felis leo*.

Ascaris sp. Glaue, 1910 (*Zool. Anz.* **35**, 748, fig. 1G), from *Felis serval*.

Ascaris sp. Glaue, 1910 (*Zool. Anz.* **35**, 748, fig. 1H), from *Felis pardochrous*.

(2) *Hosts*

As it was the prevalent opinion during the latter part of the nineteenth century that the ascaridids of dogs and cats belong to the single species, *Ascaris mystax*, it is virtually impossible to determine the host-range of *Toxocara cati* from these records, particularly with regard to its occurrence in the Canidae. It was not until Railliet & Henry (1911) clarified the situation by differentiating the morphological features of *T. canis*, *T. cati* and *T. leonina*, that host records became reliable. Since that time there have been many surveys of the parasites of dogs and cats in various parts of the world, and the writer can find no certain instance of the occurrence of *T. cati* in Canidae. It appears likely that *T. cati* is restricted to the Felidae, although Mendheim, Scheid & Schmidt (1951) state that this species has been recorded eighteen times from man, and consequently it must be regarded also as an occasional human parasite; it has been recorded once as *Toxocara crenulata* from an aoudad (*Ovis tragelaphous*) by Canavan (1929). A list of the known natural hosts of *Toxocara cati* is given in Table 1.

(3) *Development*

There have been several studies on the development of '*Ascaris mystax*', but in most cases the parasite proved not to be *Toxocara cati*. Thus Nelson (1852) described in detail the process of egg formation, fertilization and early embryonation in '*Ascaris mystax*'; but his illustrations show that his material was *Toxascaris leonina*. Hering (1873) and Stewart (1918) studied the development of '*Ascaris mystax*', but their material was of canine origin. Leuckart (1876) appears to have been the first to study the development of *Toxocara cati*; he attempted unsuccessfully to infect cats by feeding the eggs, but he made many observations on the

* Railliet & Henry (1911) re-examined *Ascaris crenulata* and found that it closely resembled *Belascaris mystax* except for the undulating cervical alae and for the longer spicules, which measured 2·1 mm. Canavan (1929), who recorded *Toxocara crenulata* from *Lynx canadensis*, stated that the former was distinguished from the latter parasite by its longer spicules which he gave as measuring 1·95–2·05 mm. However, these measurements are all within the range of spicule length measurements given for *Toxocara cati* by Yamaguti (1935) and Kreis (1938), and by the writer in the present paper (Table 8). The writer has compared specimens from *Lynx canadensis* and *Felis catus*, and found no difference in the length of the spicules and observed that the cervical alae in the parasites from both hosts occasionally appear crenulated at the edges; he takes the view that this character is of no specific value.

development of the larvae in natural infections. He observed the larvae in the stomach, that they undergo a moult and that they pass into the intestine. He described the changes which take place before the adult stage is reached. Sarles & Stoll (1935) attempted to infect cats by feeding eggs of *T. cati*, but although the larvae were shown to migrate through the lungs of rodents, they obtained only very low-grade infections in cats. They found that the first moult occurred in the egg 17 days after culture, but that they were not infective to rodents until 20 days. They found that hatching of the eggs occurred mostly in the stomach of cats within 3 hr. of ingestion.

Table 1. *List of natural hosts of* Toxocara cati

Host	Common name	Species cited	Author
Acinonyx jubatus	Cheetah	*Belascaris mystax*	Vevers (1923)
Felis bengalensis	Leopard cat	*B. mystax*	Baylis & Daubney (1922)
F. catus (type host)	Domestic cat	*Ascaris teres*	Goeze (1782)
F. chaus	Indian wild cat	*Belascaris mystax*	Baylis & Daubney (1922)
F. concolor	Cougar	*Ascaris leptoptera*	Leidy (see Walton, 1927)
F. jaguarondi	American tiger	*A. canis*	Smith (1908)
F. leo	Lion	*Toxocara mystax*	Kreis (1938)
F. macroura	Long-tailed tiger cat	*Ascaris leptoptera* var. *crenulata*	Diesing (1851)
F. maniculata	Kaffir cat	*A. mystax*	Diesing (1851)
F. moormensis	Moormikatze	*A. globulus*	von Linstow (1899)
F. nebulosa	Clouded leopard	*Belascaris mystax*	Vevers (1923)
F. nigripes	Black footed cat	*Ascaris mystax* var. *felis*	Gough (1909)
F. onca	Jaguar	*A. crenulata*	Bremser (1824)
F. pardalis	Mexican ocelot	*Toxocara mystax*	Canavan (1931)
F. pardus	Leopard	*Belascaris mystax*	Baylis & Daubney (1922)
F. planiceps	Little Malayan red cat	*B. mystax*	Cameron (1928)
F. serval	African serval	*Belascaris* sp.	Henry & Joyeux (1920)
F. silvestris	European wild cat	*Toxocara mystax*	Baylis (1939)
F. tigrinum	Tiger cat	*T. mystax*	Thwaite (1927)
F. tigris	Asiatic tiger	*Belascaris mystax*	Baylis & Daubney (1922)
F. viverrina	Asiatic wild cat	*B. mystax*	Baylis & Daubney (1922)
Lynx baileyi	Bay lynx	*Ascaris* sp.	Hall (1912)
L. canadensis	Canadian lynx	*Toxocara mystax*	Canavan (1931)
L. lynx	European lynx	*T. mystax*	Kreis (1938)
L. rufus	American bobcat	*T. mystax*	Canavan (1931)
L. rufus texensis	Spotted lynx	*T. mystax*	Canavan (1931)

II. METHODS

Cats which had been destroyed at a local cats' refuge were collected twice weekly over a period of 6 months (January to July). The intestinal contents were washed out and the wall lightly scraped. The material was placed in muslin suspended by an elastic band over the top of a beaker of physiological saline and left for 2 hr. at

37° C. The deposit was examined for larvae at a magnification of twenty-one times, and the material remaining in the muslin was searched with the dissecting microscope. Eggs of *T. cati* were obtained by dissection of the worms obtained from these cats; they were cultured at room temperature in moist charcoal for 28 days before being used. Mice were infected by suspending the eggs in water and mixing with powdered meal in a small dish, each mouse being isolated until the infected meal was eaten. Cats were infected by giving them eggs poured over meat, also by allowing them to eat the tissues of experimentally infected mice. The stomach and intestinal wall, the liver, lungs, and muscular tissues of cats were ground and digested in a pepsin solution (1 g. pepsin to 100 g. tissue), the pH being adjusted to 1·0 with hydrochloric acid. Digestion was continued for 18 hr. at 37° C., the material being then strained, and poured into a cylinder for sedimentation for half an hour. The sedimentation was repeated until the material was clear enough for examination of the deposit for larvae. Mice and rats were trapped in various localities, they were skinned, ground and digested in pepsin (1 g. per mouse) at pH 1·0 for 3 hr.; the digest was strained, poured into a cylinder for sedimentation and searched for larvae. Experimentally infected mice were treated in the same manner, except that their component tissues were digested separately as described previously (Sprent, 1952*a*). Worms were fixed in hot 3% formalin or 70% alcohol; larvae were cleared by evaporation in 5% glycerine in 70% alcohol; adults were cleared in beechwood creosote. Larvae were stained by the method previously described (Sprent, 1953). Cats and dogs used for experimental infection were kept for at least 1 week before infection; their faeces were examined for nematode eggs and they were given 50 mg. diethylcarbamazine acid citrate ('Banocide') on 2 successive days.

III. EXPERIMENTAL PROCEDURE AND OBSERVATIONS

(1) *Experimental infections*

(*a*) *Invertebrates*

Faeces from a cat experimentally infected with adult *T. cati* were mixed with equal parts of water, moist earth and sawdust. This material was placed in a terrarium containing earthworms, beetle larvae, woodlice, cockroaches and mice. After a period of 4 weeks the animals were removed, and their tissues were searched for larvae. Second-stage larvae of *T. cati* were recovered from the tissues of earthworms, cockroaches and mice.

(*b*) *Mice*

A series of thirteen white mice was infected by feeding 5000 eggs; one mouse was killed on each successive day for 13 days after infection. Various digested tissues and the contents of the alimentary canal were searched for larvae; numbers of larvae counted in the tissues are shown in Table 2. The table shows that most of the larvae after the third day were distributed to the general musculature as has previously been found to occur in mice infected with larvae of *T. canis* (Sprent, 1952*a*). Only a single larva was found in the stomach and intestinal contents of these mice, demonstrating an almost exclusively somatic type of migration. A second and

third series of thirteen mice were infected in the same manner; there was no essential difference in the distribution and appearance of the larvae in the three series. Haemorrhages were observed on the dorsal surface of the brain for the first 6 days after infection and on the lungs for the first 8 days; there were white streaks on the liver for the first 2 days. The average length and range of lengths of larvae in the tissues of mice from 1–13 days after infection are given in Table 3.

Table 2. *Distribution of larvae in tissues of mice fed* 5000 *eggs of* Toxocara cati

Days after infection	Carcass (front)	Carcass (back)	Liver	Lung	Kidney	Brain	Intestinal wall	Stomach wall	Stomach and intestinal contents
1	0	1	202	10	0	0	7	2	0
2	26	24	48	100	5	2	42	1	0
3	149	73	0	20	0	1	2	1	0
4	211	127	3	3	1	0	0	2	0
6	165	115	0	2	0	0	1	1	0
7	119	72	0	0	0	0	1	0	1
8	214	121	20	0	0	0	1	1	0
9	1072	300	0	1	0	0	0	0	0
11	52	21	0	0	0	0	0	0	0
12	271	121	15	0	0	1	0	1	0
13	202	58	0	0	0	0	0	2	0

Table 3. *Lengths of larvae in tissues of mice* (1–13 *days after infection*)

Days after infection	Average length (mm.)	Length range (mm.)
1	0·378	0·306–0·423
2	0·398	0·365–0·423
3	0·413	0·382–0·429
4	0·408	0·388–0·429
5	0·420	0·394–0·458
6	0·412	0·388–0·435
7	0·421	0·400–0·441
8	0·418	0·394–0·435
9	0·401	0·400–0·429
10	0·394	0·370–0·435
11	0·405	0·388–0·429
12	0·421	0·406–0·441
13	0·419	0·394–0·453

(*c*) *Cats*

Cats were infected by two methods: (1) by feeding eggs mixed with the food, and (2) by feeding infected mouse tissues. Twenty cats were infected by the former method and thirteen cats were infected by the latter method; they were killed at various intervals after injection, and various tissues were searched for larvae. In comparing the migration and development of the larvae in egg-infected and mouse-infected cats, cats were chosen in such a way that in each litter of kittens half were given eggs and half were given mice. In this way, by means of ten such pairs of kittens, it was possible to compare the distribution and development of the larvae at 1, 2, 3, 6, 10, 13, 21, 28, 35 and 42 days respectively after each method of infection. The distribution of the larvae in various tissues of these kittens is shown in

Table 4. For the infection of these pairs of kittens, the same culture of eggs was used for direct infection of one kitten as was used to infect the mice given to the other kitten; 10,000 eggs were given to the former, and two mice, each having been given 5000 eggs, were given to the latter.

Table 4. *Distribution of larvae in tissues of egg- and mouse-infected cats*

Days after infection	1	2	3	6	10	13	21	28	35	42	
Cat number ...	C63	C64	C42	C43	C44	C45	C56	C70	C71	C72	
Lungs	0	0	1	74	66	12	56	28	19	10	Infection with eggs
Muscle	0	0	0	0	2	3	23	3	4	3	
Intestinal wall	0	8	4	0	0	0	60	15	0	0	
Stomach wall	3	9	5	17	6	18	190	31	30	5	
Intestinal contents	0	0	0	0	0	0	73	55	260	183	
Stomach contents	0	0	0	0	0	12	215	130	4	2	
Cat number ...	C60	C61	C51	C52	C53	C54	C57	C73	C74	C75	
Lungs	0	0	0	0	0	5	6	0	0	0	Infection with mice
Muscle*	0	0	0	0	0	0	1	0	0	0	
Intestinal wall	0	6	0	3	2	31	48	3	0	0	
Stomach wall	178	182	108	490	239	234	37	10	0	0	
Intestinal contents	0	0	0	0	0	0	114	178	84	278	
Stomach contents	10	0	0	0	52	548	60	29	0	32	

* Amount of muscle examined was approximately 15% of carcass.

The mice used to infect the cats shown in Table 4 had been infected with eggs 2–6 weeks before they were fed to the cats, except for C61 which was fed with mice infected for 7 days.

A pregnant cat was infected during the last 4 weeks of gestation by three infections of 10,000 eggs at weekly intervals. Three kittens were born on the day after the last infection. Two were killed when 3 days old and one when 4 days old; the tissues and contents of the alimentary tract were searched for larvae. Apart from one second-stage larva found in the muscle tissue of a 3-day-old kitten, all were found to be uninfected.*

In some instances in tracing the migration of the larvae certain tissues not shown in the tables were examined, namely blood, brain, liver and tracheal washings.

(d) Dogs

Four recently weaned puppies were infected by feeding 10,000 eggs of *T. cati*; they were killed at 1, 2, 3 and 4 weeks after infection. The lungs, muscles, and wall of the alimentary tract were digested; these tissues and the contents of the alimentary tract were searched for larvae. Table 5 shows that larvae were found in the lungs and muscular tissue, but no larvae appeared in the wall or contents of the stomach and intestines. Numerous haemorrhagic spots were present on the lungs at 7–21 days (Pl. I, fig. 15). In so far as the larvae migrated into the somatic tissues, none appearing in the alimentary tract, the behaviour of larvae in these dogs

* A similar experiment was carried out on a pregnant bitch with eggs of *T. canis* and all the puppies were found to harbour many larvae of *T. canis* in the lungs and alimentary tract.

resembled that occurring in mice. Two puppies were each given two mice infected previously with eggs of *T. cati*, but no larvae were found in the tissues or in the contents of the alimentary tract when they were killed 3 weeks after infection.

Table 5. *Distribution of larvae of* Toxocara cati *in egg-infected dogs and sheep*

	D46	D47	D48	D49	Sheep	Sheep
Duration of infection (weeks) ...	1	2	3	4	10 (days)	17 (days)
Lungs	6	0	8	0	19	60
Muscle*	71	10	27	3	1	0
Stomach wall	0	0	0	0	—	—
Stomach contents	0	0	0	0	—	—
Intestinal wall	0	0	0	0	—	—
Intestinal contents	0	0	0	0	0	0

* The amount of muscle tissue was approximately 15% of the carcass in the case of dogs and 5% for sheep.

(*e*) *Lambs*

Two 3-month-old lambs were infected by feeding two doses of 10,000 eggs with an interval of 6 days between. They were killed 4 and 11 days after the last infection respectively. The lungs, intestinal contents and pieces of muscle were examined for larvae. Larvae were found in the lungs and muscles (see Table 5).

(*f*) *Chickens*

Four week-old chicks were given 5000 eggs mixed with mash and killed at 10, 15, 20 and 25 days. The intestinal wall, liver, lungs and carcass were digested, and searched for larvae. Six larvae were found in the carcass of the chick killed at 10 days, but they were not found in the other chicks.

(2) *Natural infections*

(*a*) *Rats and mice*

Mouse and rat traps were set at night in the vicinity of the Veterinary School; rats were also obtained from the Health Department of the Brisbane City Council. A total of twenty-two rats and four mice were digested and the tissues searched for larvae. Larvae of *Toxocara* spp. were recovered in only one animal—a rat.

(*b*) *Cats*

Over a period of 6 months a total of 260 cats were examined for infection with ascaridid nematodes. Table 6 shows the incidence of infection. It will be observed that kittens were more frequently infected; the total incidence in all ages was 34%. All specimens recovered were *T. cati*, no natural infections with *T. canis* or *Toxascaris leonina* being detected in cats.

Table 6. *Incidence of* Toxocara cati *in* 260 *cats*

Age group	Number examined	Percentage infection
Adults	175	25
Young adults	69	48
Kittens	16	69

A total of seventeen kittens removed from the uterus at Caesarean section were searched for larvae. In seven the liver, lungs and intestinal contents only were examined, in ten the whole kitten was digested. In none of the kittens were any larvae found.

(3) *Growth and development of larvae*

As indicated previously, cats were successfully infected by two methods, i.e. by feeding eggs and by feeding mouse tissues containing larvae. The migration and development of the larvae differed according to the method of infection, as is outlined below.

In cats infected by feeding eggs, the larvae were found to migrate away from the alimentary tract before commencing development (Table 4). For the first 2 days the larvae were only found in the wall of the stomach and intestine (length 0·368–0·458 mm.). At 3 days, larvae which had undergone no structural changes, were found in the liver and lungs (length 0·353–0·382 mm.); there were also larvae in the

Table 7. *Measurements (mm.) of adult stage of* Toxocara cati *at various stages of growth*

	Length	Width	Alae (length)	Oeso-phagus (length)	Ventri-culus (length)	Vulva (from ant. end)	Spicules (length)
Females	6·4	0·145	5·740	0·763	0·086	2·051	—
	9·5	0·141	7·002	1·037	0·115	2·867	—
	15	0·216	7·768	1·341	0·135	4·632	—
	25	0·369	1·538	2·570	0·216	—	—
	45	0·495	1·891	2·840	0·315	10·073	—
	60	0·675	2·173	3·419	0·360	15·000	—
	100	1·289	3·111	5·391	0·585	20·000	—
Males	12	0·189	—	1·268	0·135	—	0·558
	25	0·405	1·622	2·240	0·200	—	1·222
	47	0·605	2·347	3·043	0·315	—	1·717
	70	1·025	2·893	4·523	0·558	—	2·311

Table 8. *Measurements (mm.) of larval stages of* Toxocara cati

Source	Larval stage	Length	Width	Excre-tory pore	Oeso-phagus (length)	Ventri-culus (length)	Tail	Genital rudiment (from ant. end)
Egg	2nd	0·369	0·015	0·063	0·113	0·017	0·036	0·220
Mouse	2nd	0·387	0·015	0·069	0·118	0·019	0·038	0·219
Earthworm	2nd	0·378	0·017	0·071	—	—	0·038	—
Cockroach	2nd	0·376	0·015	0·069	—	—	0·040	—
Cat lung	2nd	0·315	0·014	0·059	0·124	0·015	0·031	0·179
Cat stomach	3rd	0·432	0·017	0·071	0·120	0·017	0·042	0·249
Cat stomach	3rd	0·648	0·023	0·092	0·160	0·022	0·046	—
Cat intestine	3rd	0·765	0·029	0·107	0·174	0·023	0·046	0·386
Cat stomach	3rd	1·017	0·036	0·105	0·187	0·025	0·069	0·522
Cat intestine	4th	1·620	0·046	0·122	0·270	0·042	0·063	1·035
Cat intestine	4th	2·104	0·059	0·136	0·324	0·042	0·069	0·934
Cat intestine	4th	4·182	0·107	—	0·504	0·059	0·143	1·395
Cat intestine	4th	3·892	0·105	0·376	0·495	0·063	0·063	—

stomach wall (length 0·360–0·382 mm.), one of which was moulting (length 0·371 mm.). At 5 days there were larvae in the lungs (length 0·318–0·359 mm.), tracheal washings (length 0·306–0·361 mm.), and stomach wall (length 0·323–0·370 mm.); the larvae in the lungs and tracheal washings possessed anterior prolongations (see below); one larva in the stomach wall was moulting (length 0·353 mm.). At 6 days there were larvae in the lungs (length 0·360–0·405 mm.) and stomach wall (length 0·341–0·359 mm.), several possessed prolongations but there was no evidence of growth. At 10 days there were many larvae in the lungs (length 0·387–0·423 mm.) and stomach wall (length 0·333–0·477 mm.); one was moulting (length 0·382 mm.). At 11 and 13 days there were many larvae in the lungs (length 0·369–0·424 mm.), stomach contents (length 0·353–0·605 mm.), stomach wall (length 0·323–0·558 mm.), and intestinal wall (length 0·415–0·659 mm.); some larvae in the stomach wall were moulting (length 0·378–0·423 mm.). At 15 days the larvae in the lungs measured 0·370–0·451 mm.; the larvae in the stomach and intestinal walls were of variable lengths (0·334–0·865 mm.), some appearing to have grown little, others being advanced third-stage larvae; many third-stage larvae had left the stomach wall and were in the stomach contents. At 19 days there were fully developed third-stage larvae in the stomach wall and stomach contents, also moulting larvae measuring 0·999–1·131 mm.; fourth-stage larvae had appeared in the stomach contents (length 1·289–1·631 mm.) and in the intestinal contents also there were many fourth-stage larvae (length 2·389–3·777 mm.). At 21 days larvae were still present in the lungs; in the stomach wall and stomach contents there were third-stage larvae in various stages of development, also moulting larvae measuring 1·053–1·262 mm.; fourth-stage larvae were present in large numbers in the stomach contents (length 1·289–1·972 mm.), intestinal wall (length 1·446–2·409 mm.) and intestinal contents (length 1·920–2·409 mm.). At 28 days there were small larvae in the muscle (length 0·378–0·393 mm.) and lungs (length 0·350–0·514 mm.); in the stomach wall there were developing third-stage larvae (length 0·350–0·786 mm.), moulting larvae (length 0·736–0·964 mm.), and a fourth-stage larva (length 1·350 mm.); in the stomach and intestinal contents there were fourth-stage larvae (length 3·555–4·339 mm.), moulting larvae (length 4·333–5·0 mm.) and adults (length 4·9–19 mm.). At 35 days there were larvae in the muscle (length 0·378–0·393 mm.) and lung (length 0·343–0·436 mm.); in the stomach wall there was a third-stage larva (length 0·421 mm.), a moulting larva (length 0·621 mm.) and a few fourth-stage larvae (length 1·143–1·664 mm.); in the stomach and intestinal contents there was a fourth-stage larva (length 2·945 mm.) and many adults (length 5·8–30 mm.). At 42 days there were still a few larvae in the muscle (length 0·378–0·414 mm.) and lungs (length 0·371–0·400 mm.); in the stomach and intestinal contents there were fourth-stage larvae (length 3·955–4·133 mm.) and many adults (length 4·8–42 mm.).

In cats infected by feeding infected mouse tissues the larvae remained mostly confined to the alimentary tract, larvae being found in the lungs of the cats killed at 13 and 21 days only (Table 4); they measured 0·420–0·540 mm. in length. On the day following ingestion of the mouse, the stomach contents and stomach wall contained many small larvae, they were indistinguishable from those observed in

mouse tissues (length 0·432–0·450 mm.). At 2 and 3 days after infection the larvae were confined to the stomach wall, there were no moulting forms, but some larvae showed slight increase in length (length 0·376–0·565 mm.). At 6 days there were many growing larvae in the stomach wall (length 0·441–0·753 mm.); many were moulting (length 0·529–0·765 mm.); in the intestinal wall there were smaller larvae (length 0·412–0·453 mm.). At 10 days there was further growth of the larvae in the stomach wall (length 0·500–1·247 mm.), several larvae being in the process of moulting (length 0·765–1·247 mm.); there were also many larvae in the intestinal wall (length 0·541–0·558 mm.) and in the stomach contents (length 0·558–1·117 mm.) and a few fourth-stage larvae had appeared (length 1·117–1·200 mm.). At 13 days there were third-stage larvae (length 0·755–1·235 mm.) and moulting larvae (length 0·630–1·235 mm.) in the stomach wall; in the stomach contents there were third-stage larvae (length 0·793–1·059 mm.), moulting larvae measuring 1·012–1·059 mm. and fourth-stage larvae (length 1·029–1·381 mm.); some of the fourth-stage larvae had migrated to the intestine, but they were recovered only from the intestinal wall (length 1·250–1·800), none being found in the contents. At 21 days there were still third-stage larvae in the stomach wall (length 0·447–1·236 mm.); in the stomach contents there were third-stage larvae (length 0·999–1·083 mm.) and fourth-stage larvae (length 2·288–2·472 mm.); in the intestinal wall there were fourth-stage larvae (length 1·656–3·813 mm.); in the intestinal contents there were fourth-stage larvae (length 3·419–5·654 mm.), and also young adults (length 5·707–7·416 mm.).

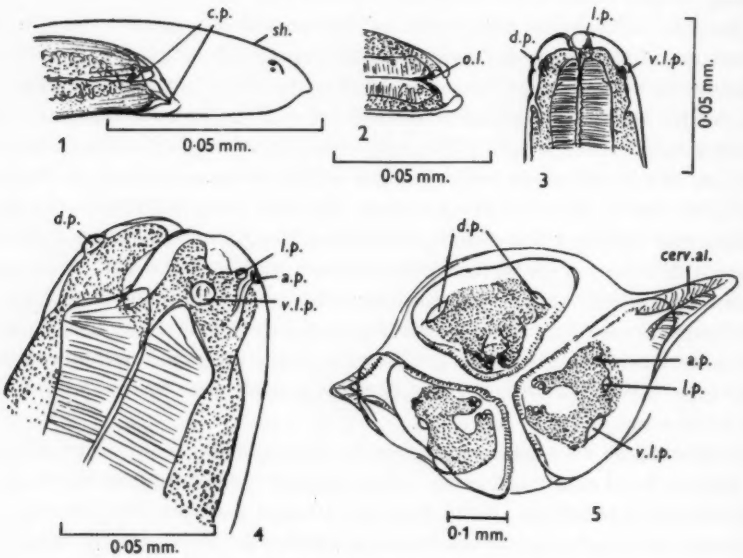
At 28 days there were fourth-stage larvae (length 3·400–5·067 mm.), a moulting larva (length 4·711 mm.) and many adults (length 5·2–19 mm.) in the contents of the stomach and intestines. At 35 days and 42 days the worms had all reached the adult stage, the length measurements being 10–35 mm. and 5–48 mm. respectively.

It was thus evident that in the egg-infected cats the larvae migrated throughout the body to the muscle tissue and lungs and eventually reached the stomach wall. In contrast, most larvae in mouse-infected cats did not migrate, but entered the stomach wall and commenced growth. There may be exceptions to this general pattern of behaviour in that some larvae may perhaps remain in the stomach wall of egg-infected cats and not undergo migration, and some of the larvae in mouse-infected cats may undergo migration—larvae were in fact occasionally found in the lungs of mouse-infected cats (Table 4). Between 5 and 14 days after infection the egg-infected cats showed haemorrhagic spots on the lungs; these were not observed in mouse-infected cats.

(a) *The second-stage larva*

At the time of hatching, the second-stage larva lies within the cuticular sheath of the first stage. Like the larvae of *Toxocara canis*, they moved more vigorously than the larvae of other mammalian ascaridids and appeared more robust when compressed. Twenty larvae removed from the eggs in this way and killed by heat, measured 0·312–0·423 mm. in length. The anterior extremity of the sheath is asymmetrical (Text-fig. 1); the mouth of the second-stage larva lying within the sheath is bounded by three indistinct lips, each with a minute papilla. In the

recently hatched larva the oesophagus, oesophageal ventriculus, intestine and rectum were clearly demarcated (Text-figs. 10, 16). In second-stage larvae recovered from host tissues these structures were often obscured by the granules which are deposited throughout the tissues of the larvae. The structure of a second-stage larva obtained from the liver of a cat 3 days after infection with eggs is shown in Text-fig. 21; its length was 0·360 mm. The cuticle at this stage showed no



Text-figs. 1–5. *Toxocara cati*.

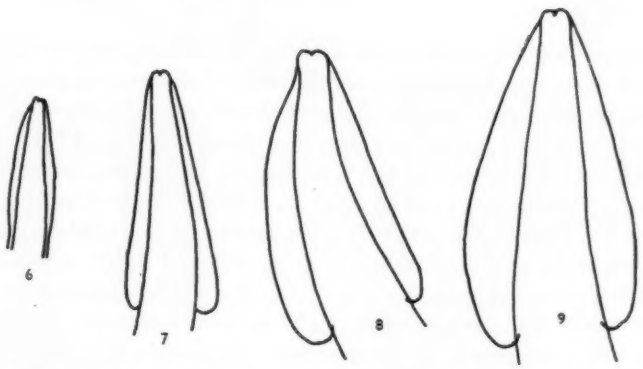
Text-fig. 1. Anterior end of second-stage larva within cuticle of first stage.
Text-fig. 2. Anterior end of third-stage larva from stomach wall of cat.
Text-fig. 3. Anterior end of fourth-stage larva from intestinal wall of cat.
Text-fig. 4. Anterior end of young adult (lateral view).
Text-fig. 5. Anterior end of adult (*en face* view).

Abbreviations for figures

a.p.	amphidial pore	*oes.*	oesophagus
an.	anus	*ph.*	phasmid
c.p.	cephalic papillae	*r.g.*	rectal gland
cerv.al.	cervical ala	*r.c.*	rectal cell
cl.	cloaca	*r.n.g.*	rectal nerve ganglion
d.p.	dorsal lip papilla	*sh.*	cuticular sheath
e.p.	excretory pore	*sp.*	spicule
g.r.	genital rudiment	*s.s.*	spicule sheath
i.p.	internal lip papilla	*ut.*	uterine branch
in.	intestine	*v.l.p.*	ventro-lateral lip papilla
l.p.	lateral lip papilla	*vt.*	ventriculus
n.r.	nerve ring	*vg.*	vagina
o.l.	cuticular lining of oesophagus	*vu.*	vulva.

corrugations, but the lateral alae were conspicuous, especially in transverse sections, commencing about mid-way between the excretory pore and the anterior end.

The eggs of *T. cati* were found to hatch in earthworms, cockroaches, chickens, mice, dogs, lambs and cats, and the larvae were recovered from their tissues. The length measurements of larvae from the tissues of mice are shown in Table 3; the maximum length attained by the larvae in earthworms was 0·378 mm., in cockroaches 0·375 mm., in chickens 0·396 mm., in lambs 0·423 mm., and in dogs 0·423 mm. In cats the maximum length attained by the larvae in the muscle tissues was 0·459 mm. Larvae recovered from tissues of experimentally infected mice manifested structural changes in the mouth region between 8 and 11 days after infection. The earliest change was the separation from the anterior tip of the oesophagus of three minute triangular plates. Later there appeared a prolongation from the mouth region which appeared as though the lining of the oesophagus was being gradually everted (Pl. I, fig. 6). In some larvae globules appeared at the



Text-figs. 6–9. *Toxocara cati*. Cervical region of adult with a length measurement of 15, 25, 45 and 60 mm. respectively.

mouth and excretory pore. The cuticle became irregular and rough so that particles adhered to it, but the actual process of moulting was not observed. The length of the larvae undergoing the above changes in mouse tissues was 0·333–0·378 mm.

The anterior prolongation was also observed in larvae from the lungs and tracheal washings of cats 5 days after infection with eggs (Plate I, figs. 4, 5), also in larvae from the stomach wall 6 days after infection with eggs. At 3, 5, 10 and 11 days after infection with eggs, small moulting larvae (length 0·353–0·382 mm.) were found in the stomach wall, and in the lungs, at 5 days, some of the larvae showed a slight loosening of the cuticle, indicating that the second moult occurs in the stomach wall and possibly also in the lungs. The significance of the oral prolongation is obscure: it was not clear whether or not it was associated with the moult.

It was evident that in egg-infected cats the larvae reached the lungs and eventually found their way to the stomach wall where growth and development commenced. Probably they reached the stomach wall via the trachea, but it is also probable that many of the larvae, after reaching the lungs, were distributed via the

arterial circulation to the muscles and connective tissues, and that some may be able to reach the tissues of the alimentary tract by this route. It was not possible to ascertain whether the larvae in the muscles and lungs of egg-infected cats had moulted and were to be regarded as second- or third-stage larvae; their appearance indicated that they were second-stage larvae. In mouse-infected cats larvae did not show oral prolongations; moulting larvae were observed in the stomach wall at 6 days after infection, their length being 0·459–0·765 mm. If these moults are to be regarded as second moults, it is evident that the second-stage larvae grow to a larger size in mouse-infected cats before the second moult.

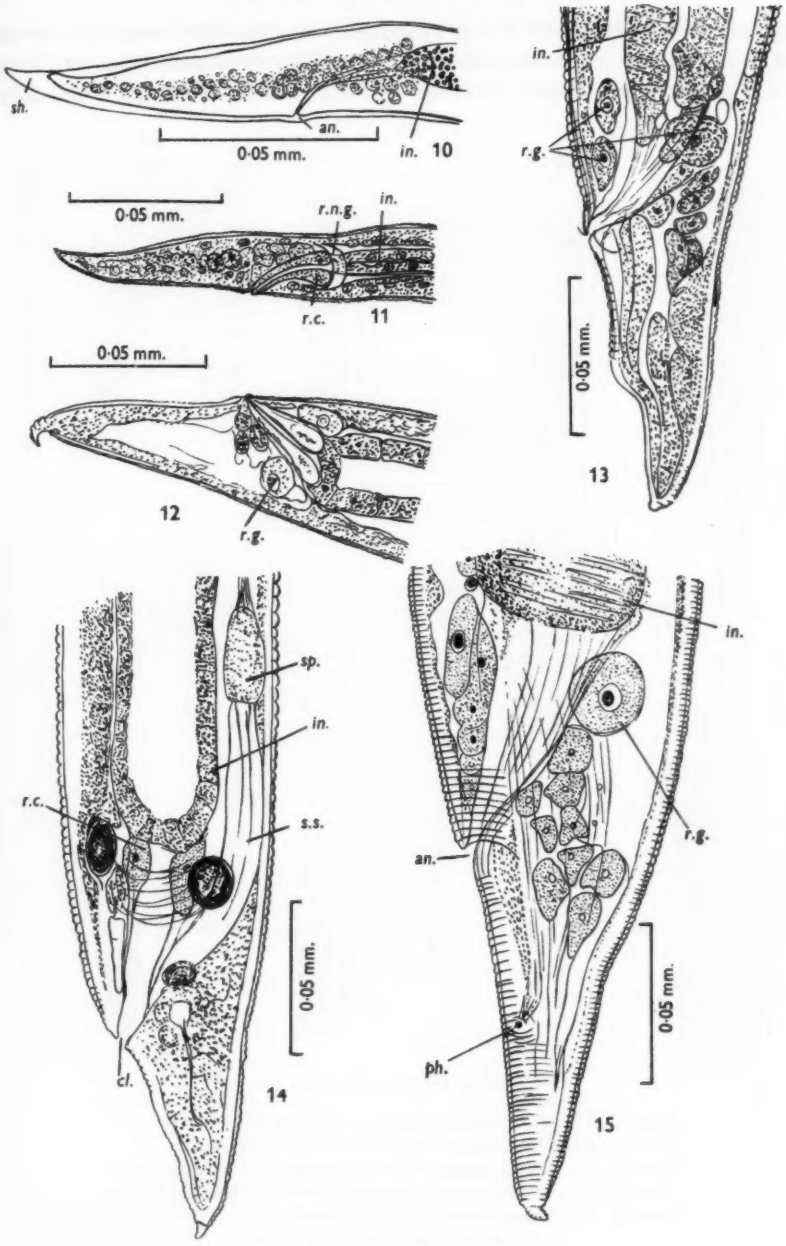
The second stage is one of active migration, the hatched larva is vigorously motile, burrows into the tissues and migrates through the body. There appeared to be very slight growth during this stage. The second stage ends with the second moult which occurs in the stomach wall, and possibly also in the lungs, of cats.

(*b*) *The third-stage larva*

Apart from the possible occurrence (see above) of third-stage larvae in the somatic tissues, which fail to develop further, development of these larvae is confined to the wall of the stomach of cats. Considerable growth occurs during this stage, the larvae growing from about 0·4 mm. (Pl. I, fig. 1) to about 1·3 mm. (Pl. I, fig. 2). The most characteristic structural change is in the oral region, the ventral lips undergoing marked development so as to form a vestibule for the mouth (Text-fig. 2; Pl. I, figs. 6, 7). There is also a considerable increase in the number of cells during this stage, especially in the intestine (Text-fig. 22) and in the regions of the tail (Text-fig. 11) and oesophagus (Text-fig. 17). During development of the third-stage larvae white nodules appeared on the mucous membrane of the stomach and also, in heavy infections, on the intestine. On section these were found to be granulomatous clusters of cells, mostly lymphocytes, containing coiled larvae in the centre (Pl. I, fig. 17); other larvae were found lying freely in the mucous and submucous layers. Many third-stage larvae leave the stomach wall and were found in the contents in various stages of development. The third moult took place when the larvae measured 0·999–1·235 mm. in length. During this moult a marked reorganization of the mouth region occurred; three triangular pieces of cuticle lining the anterior end of the oesophagus were discarded together with the oesophageal lining, and the lips of the fourth-stage larva assumed the characteristic ascaridid appearance (Pl. I, fig. 8).

(*c*) *The fourth-stage larva*

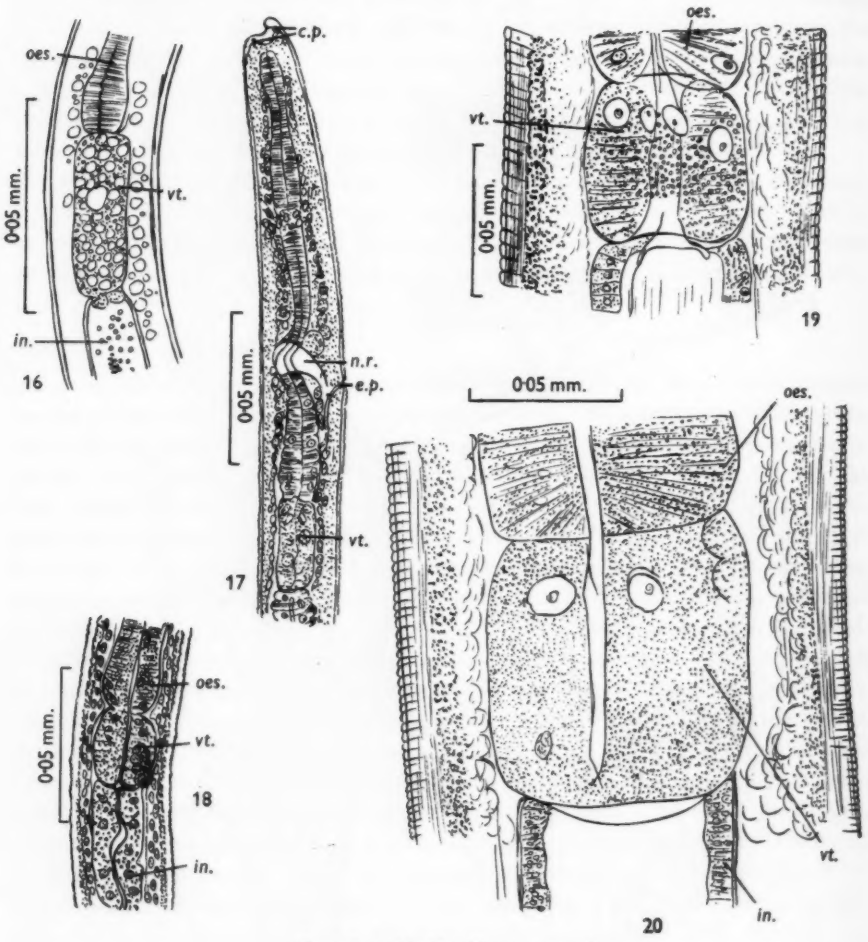
Whereas the principal location for the development of the third-stage larva was the stomach wall, the fourth-stage larvae occurred in the stomach contents, intestinal wall and intestinal contents. In egg-infected cats fourth-stage larvae were first observed at 19 days; the smallest specimens occurred in the stomach contents, measuring 1·289–1·631 mm. in length. In mouse-infected cats fourth-stage larvae (length 1·117–1·200 mm.) were found in the stomach contents at 10 days.



Text-figs. 10–15. *Toxocara cati*.

Text-fig. 10. Tail of second-stage larva within cuticle of first stage.
Text-fig. 11. Tail of third-stage larva from stomach wall of cat.
Text-fig. 12. Tail of young fourth-stage larva from stomach contents of cat.
Text-fig. 13. Tail of female fourth-stage larva from intestine of cat.
Text-fig. 14. Tail of male fourth-stage larva from intestine of cat.
Text-fig. 15. Tail of adult female from intestine of cat.

The fourth-stage larva (Pl. I, fig. 3) differed from the third-stage larva in that the lips had acquired the characteristic ascaridid appearance; the oesophageal ventriculus was more globular (Text-fig. 18); the intestine possessed a well-defined

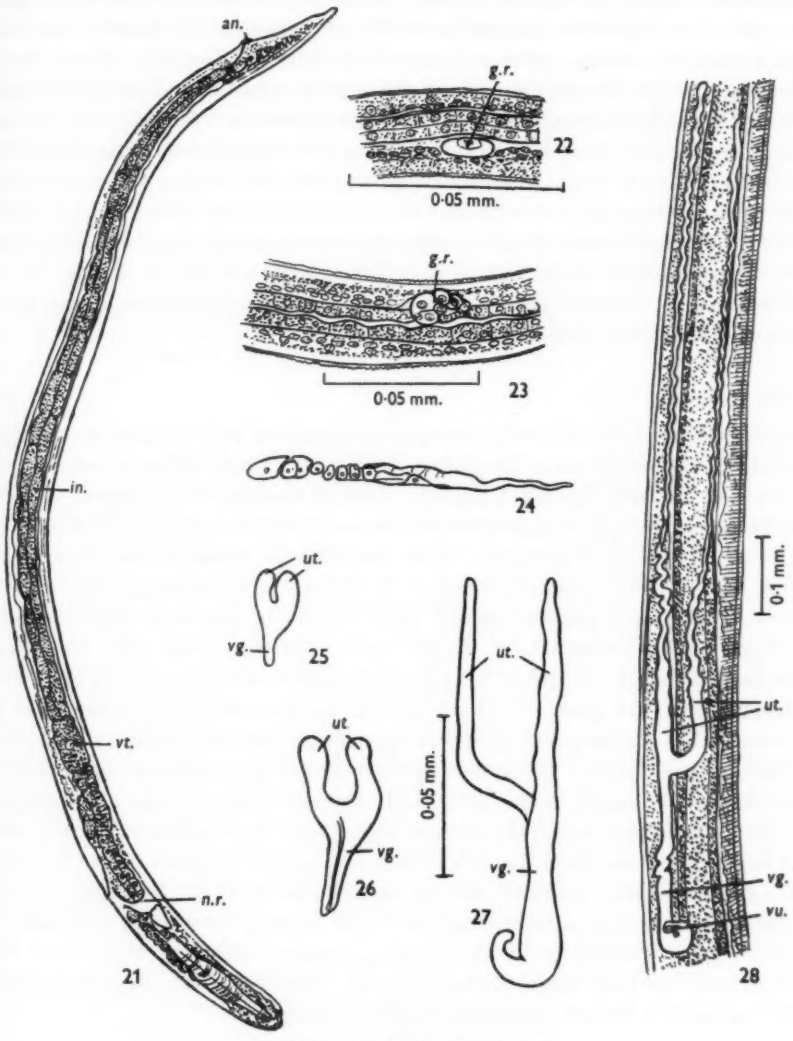


Text-figs. 16–20. *Toxocara cati*.

Text-fig. 16. Oesophageal region of second-stage larva.
Text-fig. 17. Oesophageal region of third-stage larva.
Text-fig. 18. Oesophageal region of young fourth-stage larva.
Text-fig. 19. Oesophageal region of older fourth-stage larva.
Text-fig. 20. Oesophageal region of adult.

lumen throughout its length; the rectum was more thickly lined by cuticle; the dorsal and ventral rectal glands were differentiated; the tip of the tail was bent dorsally (Text-fig. 12). The smallest fourth-stage larvae were found in the stomach contents, but as growth proceeds they evidently pass into the intestine. At first

they are associated with the intestinal wall, but with further growth the parasite becomes free in the intestinal lumen where it undergoes considerable growth during which sexual differentiation proceeds. During the second and third stages no



Text-figs. 21–28. *Toxocara cati*.

Text-fig. 21. Second-stage larva from liver of cat.
Text-fig. 22. Genital rudiment of second-stage larva.
Text-fig. 23. Genital rudiment of third-stage larva.
Text-fig. 24. Genital rudiment of male fourth-stage larva, length 1·578 mm.
Text-fig. 25. Genital rudiment of female fourth-stage larva, length 1·315 mm.
Text-fig. 26. Genital rudiment of female fourth-stage larva, length 2·174 mm.
Text-fig. 27. Genital rudiment of female fourth stage larva, length 2·446 mm.
Text-fig. 28. Genital rudiment of young adult female, length 6 mm.

changes except cell division were observed in the genital rudiment (Text-fig. 22, 23) and at the third moult it had the form of a compact cluster of cells. During the fourth stage cellular multiplication proceeds further so that sexual differentiation was evident at a length of about 1·5 mm. In the female larva the genital rudiment developed three digitiform extensions which progressively lengthened and eventually formed the vagina, uteri and ovaries as shown in Text-figs. 25–28. In the male larva a single filament grew from the posterior end of the genital rudiment (Text-fig. 24) and the developing spicules became evident (Text-fig. 14). The tail of the female fourth-stage larva (Text-fig. 13) was more slender than that of the male larva (Text-fig. 14). Considerable growth occurred during the fourth stage, and as growth proceeded the cuticular corrugations became wider; by this feature it was possible to differentiate the fourth-stage larvae clearly from the young adult stage whose cuticular corrugations were much narrower (cf. Pl. I, figs. 13, 14). The fourth moult occurred in the intestinal contents; moulting fourth-stage larvae measured 4·333–5·654 mm. (Pl. I, figs. 11, 12).

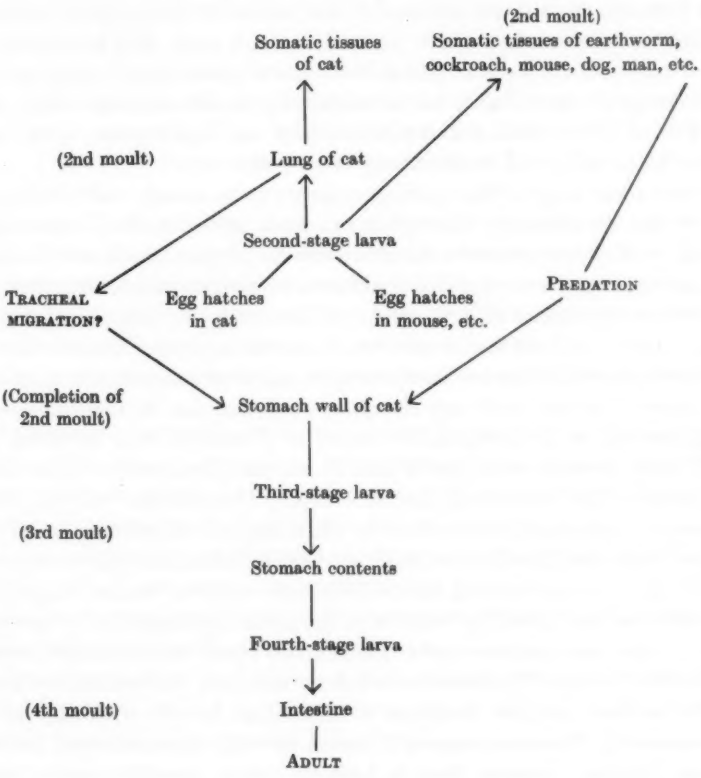
(*d*) *The adult stage*

During the fourth moult further reorganization of the buccal region was evident; three digitiform extensions of the palp extend forward and enclose a central cavity so as to form the three lips (Pl. I, fig. 10). Each of the two ventral lips of the adult stage have a single large oval papilla on the ventro-lateral aspect, and a similar but smaller papilla situated laterally. Near, but slightly dorsal to the latter, is the minute canal of the amphid (Text-figs. 4, 5). The dorsal lip possesses two large oval papillae. These large papillae appear more simple in structure than the corresponding papillae possessed by *Ascaris devosi* (Sprent, 1953). The dentigerous ridges were visible at a length of 9·5 mm. The adult female possesses an open vulva and the tail bears two phasmids (Text-fig. 15). In the male the spicules appear, the tail assumes the characteristic probular appearance, and the preanal and postanal papillae become visible. Recently moulted adults have lateral alae extending almost the whole length of the body, but as growth proceeds the alae disappear from the posterior end and in the cervical region they become wider. The alae of the adult stage commence from the ventral lips, whereas in previous stages they commence about midway between the lips and the nerve ring. Gradually the alae become shorter, and, at a body length of about 25 mm. they begin to assume the arrow shape characteristic of the mature parasite, although this is not fully achieved until the body length is about 60 mm. (Text-figs. 6–9). Eggs were present in the vagina of a female measuring 55 mm. in length.

IV. DISCUSSION

It has been generally assumed that the life history and development of *Toxocara cati* is identical with that of *Ascaris suum*. Evidence has been presented in this paper which indicates that this assumption is incorrect. The development of *A. suum* was described by Roberts (1934); he reported that the second and third moults occurred in the lungs of the pig and the fourth moult in the intestinal

contents; he did not mention any phase of development in the wall of the alimentary tract. Wright (1935) observed that the second-stage larvae of *Toxascaris leonina* developed and moulted in the intestinal wall of dogs and that the third-stage larvae returned to the lumen without lung migration being necessary; the third and fourth moults occurred in the intestine. Sprent (1953) found that the larvae of *Ascaris devosi*, a parasite of the fisher, marten, mink and weasel, migrate into the tissues of mice where the second-stage larvae grow and the second moult occurs. The third-stage larvae are encapsulated in the tissues and await ingestion by the definitive host in whose intestine the third and fourth moults occur.



Text-fig. 29

A schematic representation of the life history of *T. cati* according to the observations presented in this paper is shown in Text-fig. 29. It is evident that with regard to its life history this parasite shares certain features shown by each of the above-mentioned species. It shares with *A. suum* the migration of the larvae to the lungs of the final host, and probably also the tracheal migration to the intestine. It shares with *Toxascaris leonina* the development in the wall of the alimentary tract, although it is the third stage of *Toxocara cati* which develops in this location. It

shares with *Ascaris devosi* the somatic migration in the tissues of rodents, which thus act as facultative intermediate hosts.

It thus seems probable that the life history of *Toxacara cati* represents a basic pattern from which the life histories of other ascaridids of terrestrial mammals have evolved. This is perhaps supported by the primitive morphological features of *T. cati*, retaining as it does the oesophageal ventriculus, a characteristic of the subfamily Anisakidae, most of which are parasites of lower vertebrates. This structure was clearly visible in the second-stage larvae, indicating that this morphological character is a primitive one, in contrast to the cervical alae which, not being developed until the young adult stage, are probably more superficial characteristics.

It has been shown that the larvae of *T. cati* will hatch and migrate in the tissues of earthworms, cockroaches, birds, mice, lambs and dogs, thus permitting a wide variety of intermediate hosts as well as direct development from embryonated eggs. This wide range of intermediate hosts enables the parasite to occur widely throughout the Felidae, from predation of ruminants by the large species, to the ingestion of insects, birds and small mammals by the smaller cats.

While the adult stage of this parasite appears to be mainly restricted to the cat family, it has occasionally been reported from other species, especially man. Mendheim *et al.* (1951) reviewed the literature on human infection with ascaridids of dogs and cats; they recorded that there have been eighteen human infections with *T. cati* and two infections with *T. canis*. Of the two latter cases, one was recorded by Leiper (1907), and one was their own observation. Leiper's case was probably *Toxascaris leonina* infection (see Introduction), and their own case also may well have been *T. leonina*, as they used only the narrow cervical alae for identification. Thus there appears to be no indisputable record of *Toxocara canis* infection in man, although there seems to be no doubt that *T. cati* must be regarded as an occasional human parasite. The first human case, recorded by Pickells (see Cobbold, 1864), may be of interest in pointing to the method by which such infections may have occurred; the subject was a woman who habitually ate the earth from the graves of priests and as a result of her unusual eating habits frequently vomited beetles, suggesting that human infection may possibly result from the accidental ingestion of beetles, cockroaches or other intermediate hosts. Preliminary observations (unpublished) have shown that the larvae of *Toxocara canis* behave similarly in intermediate hosts, but, except for newborn puppies, attempts to infect dogs directly with eggs have so far been unsuccessful. The occurrence of *Toxocara* larvae in human tissues, as described by Beaver, Snyder, Carrera, Dent & Lafferty (1952), possibly results from accidental ingestion of eggs of *T. canis* or *T. cati*, man acting in this instance as an intermediate host.

V. SUMMARY

1. An account is given of the history, synonymy and host-range of *Toxocara cati*.

2. Eggs of *T. cati* were fed to various animals, and the second-stage larvae were found in the tissues of earthworms, cockroaches, chickens, mice, dogs, lambs and cats.

3. Cats were successfully infected by feeding eggs of *T. cati* and by feeding mice harbouring larvae in the tissues.

4. In egg-infected cats the larvae were found in the liver, lungs, muscles and tracheal washings as well as in the digestive tract, indicating that they migrate through the tissues.

5. In mice the larvae were found in the liver, lungs and muscles, but did not reach the alimentary tract. Changes occurred in the mouth region of the larvae in mouse tissues between 8 and 11 days after infection, but no actual moult was observed. No significant growth of the larvae was observed in mouse tissues.

6. In mouse-infected cats the larvae were mostly confined to the wall and contents of the digestive tract.

7. It appeared that the second moult occurred in the stomach wall of egg-infected cats at 3–10 days after infection; moulting larvae measuring 0·353–0·423 mm. in length; in a mouse-infected cat moulting larvae (length 0·459–0·765 mm.) were observed at 6 days after infection.

8. In both egg- and mouse-infected cats the third-stage larvae grew in the stomach wall and moulted for the third time at a length of 0·9–1·2 mm. This occurred at about 10 days in mouse-infected cats and at about 19 days in egg-infected cats.

9. The smallest fourth-stage larvae were found in the stomach contents whence they passed into the intestine. Probably they are at first attached to the wall and later become free in the intestinal contents.

10. The fourth-stage larvae have lips resembling the adult; sexual differentiation occurs during this stage, being first evident at 1·5 mm.

11. The fourth moult occurs in the intestine at a length of approximately 4·5–5·5 mm.

12. In the adult stage the lateral alae gradually disappear and the cervical alae reach the adult form at a length of approximately 45 mm. The smallest female observed containing eggs in the vagina measured 55 mm. in length. Eggs were first observed in the faeces at 56 days after infection by eggs.

13. The evidence from experimental and natural infection indicated that infection of cats with *T. cati* always takes place after birth.

14. No larvae were found in the alimentary tract of dogs following experimental infection either with eggs or with infected mice.

15. The relationship of the life history of *T. cati* to that of other ascaridid species, to the feeding habits of the definitive hosts, and in relation to the occurrence of human infection with this parasite is discussed.

The writer's thanks are due to his wife for rearing many of the kittens used in these experiments, and to Mr S. C. Ellis and Mr J. J. Mines for their able technical assistance. The writer also wishes to acknowledge the help in obtaining certain references that he has received from Prof. R. T. Leiper, F.R.S., Director of the Commonwealth Bureau of Agricultural Parasitology, St Albans, England, and from Mr Allen McIntosh of the Agricultural Research Service of the United States Department of Agriculture. The work was supported by a research grant from the University of Queensland.

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EXPLANATION OF PLATE I

Fig. 1. Second-stage larva from tissues of mouse.
Fig. 2. Third-stage larva from stomach contents of cat.
Fig. 3. Fourth-stage larva from intestinal contents of cat.
Figs. 4, 5. The second moult in lung of cat.
Fig. 6. Larva with oral prolongation from tissues of mouse.
Fig. 7. Anterior end of third-stage larva.
Fig. 8. Third moult in stomach wall of cat.
Fig. 9. Anterior end of fourth-stage larva.
Fig. 10. The fourth moult.
Fig. 11. The fourth moult, tail of female.
Fig. 12. The fourth moult, tail of male.
Fig. 13. Cuticle of fourth-stage larva.
Fig. 14. Cuticle of adult.
Fig. 15. Lung of dog 7 days after infection.
Fig. 16. Lung of cat 5 days after infection.
Fig. 17. Stomach wall of cat 14 days after infection.

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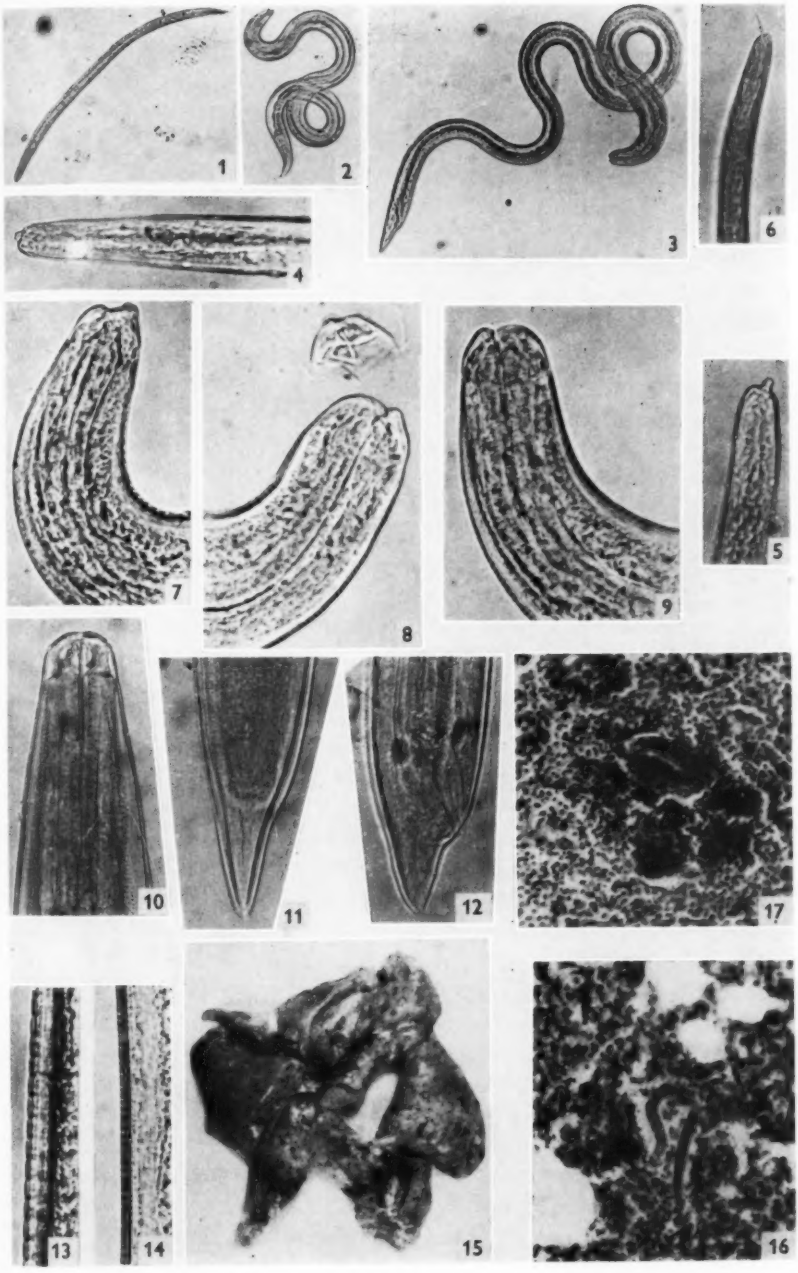

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STUDIES IN HISTOMONIASIS

I. THE INFECTION OF CHICKENS (*GALLUS GALLUS*) WITH HISTOMONAD SUSPENSIONS

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Several methods which have been employed for infecting turkey poults and hen chickens with *Histomonas meleagridis* have been summarized by Farmer & Stephenson (1949). Infections have been obtained by the injection of cultures of the protozoan *per os* and *per cloaca*, the administration of infected caecal and liver material by the same routes, the rearing of turkeys on infected ground and the running of turkeys with fowls. A commonly used method for producing experimental histomoniasis is by the administration of *Heterakis* ova. Bishop (1938) obtained caecal lesions in hen chickens by inoculating, *per os* or *per cloaca*, cultures of *Histomonas meleagridis* isolated from the liver of a hen suffering from histomoniasis. Success of transmission appears to depend on the age of the culture used; old cultures that have been subcultured for 3–4 months before inoculation may give poor results, whereas good positive results are obtained with freshly isolated cultures. On the other hand, Bishop's strain was cultured for 6 months without evidence of attenuation. Where large numbers of birds are being used for experimental purposes the maintenance of adequate supplies of fresh cultures involve much labour and time. Tyzzer & Collier (1925) inoculated young turkeys *per os* with lesions obtained from livers of birds suffering from blackhead, and seven of the eighteen birds so treated became infected and succumbed between the eleventh and fifteenth days after inoculation. Cloacal injection of similar material produced infections in sixteen of twenty birds which died 10–20 days later. Tyzzer (1934) found that the cloacal route was more reliable than the oral route in producing infections. De Volt & Davis (1936) used protozoan cultures by both routes and produced infections in 18- to 23-day-old poults and typical caecal and liver lesions developed within 10–14 days in twenty-nine of thirty-three birds so treated. Delaplane (1932) inoculated twenty-one chickens *per cloaca* with ground-up liver and caecal lesions, and thirteen developed lesions, but in only three were liver lesions developed. Bishop found that the strain she obtained from the liver of a hen which died of blackhead failed to produce liver lesions in experimentally infected chickens.

Smith (1954) investigated the effect of variations in the diet in the induction of *Salmonella gallinarum* infections. He found that different infection rates were obtained when the organisms were administered in different kinds of food which influenced the bactericidal action of the gastric juice to different extents. He considered that at least two properties of foodstuffs appeared to influence the bactericidal mechanism, their capacity to neutralize gastric acidity and their consistency. He found that foods containing fish meal raised the pH of the gizzard contents

with a consequent decrease in the destruction of *S. gallinarum* so that a greater number of bacteria passed into the duodenum. In view of Smith's observations it was decided to investigate the possible artificial infection of chickens from two stand-points: (1) by infecting starved chickens with tissue phases of *Histomonas meleagridis* (starving ensured that the protozoa would pass quickly through the gizzard into the duodenum); and (2) to produce an alkaline environment in the gizzard of starved chickens by administering an alkaline mixture immediately before infecting them with similar material.

The present paper describes the results obtained by this technique. In a further paper it is hoped to discuss the influences of various diets on the course of experimentally induced histomoniasis.

MATERIAL AND METHODS

Chickens. Barred Rock × Brown Leghorn and White Leghorn chickens aged from 1 to 3 weeks were used in these experiments. Unless otherwise stated it can be assumed that BR × BL chicks were used.

Food. The mash used was the standard ration used on the Trust's poultry plant. The formula is as follows:

	%		%
Ground wheat	20	Decorticated groundnut meal	5
Ground barley	20	Dried grass meal	5
Sussex ground oats	10	Dried skim milk	5
Maize meal	11¾	Unextracted dried yeast	2
Middlings	15	Minerals	1¼
White fish meal	5		

This mash was supplemented with the following:

Vitamin A (synth.)	2000 i.u./lb.
Vitamin D_3	450 i.u./lb.
Vitamin B_2	1 mg./lb.

Source of infective material. Young chicks of 1–3 weeks of age were infected *per os* with 500–1000 embryonated *Heterakis* ova. The chicks were sacrificed 10 days later when the caecal walls were heavily populated with invasive forms of *Histomonas*. The livers of these chickens usually showed only a few early lesions. The caeca, which were removed, provided the infective material for each experiment.

Preparation of infective material. The infected caeca were removed and cut up into small pieces which were then ground up in a mortar with fine washed silver sand and a little Ringer's solution of modified formula: NaCl, 9·0 g.; KCl, 0·2 g.; $CaCl_2$, 0·2 g. in 100 ml. distilled water. The Ringer's solution was added at a temperature of approximately 37° C. The suspension was then squeezed through cheese cloth into a beaker. By this means large fragments of tissue and debris were eliminated and a suspension of histomonads obtained. Measured quantities (1 ml.) of the suspension were pipetted into the crop of experimental chickens which were kept at hand so that there was little delay in infecting them.

Treatment of chickens to be infected. Groups of chickens were: (1) kept on normal feed to the time of infection, or (2) starved for 18 hr. before infection, or (3) starved

for 18 hr. before infection and followed by 1 g. alkali mixture administered immediately before they were infected, or (4) kept on normal feed to time of infection when they also received 1 g. alkali mixture. All groups had free access to water throughout the experiments.

The alkali mixture. The mixture consisted of calcium carbonate—40 %, magnesium trisilicate—17 %, colloidal kaolin—43 %. One gram of the mixture was administered to each chicken in 1·0–1·5 ml. distilled water 5–15 min. before infection with caecal filtrate.

Assessment of infections. Although chickens are susceptible to infection with *Histomonas meleagridis* they rarely die as a result, and it was therefore necessary to sacrifice chickens at about 7–14 days after the administration of the histomonad suspension. Examinations post-mortem were made to determine the extent to which the caeca and livers were affected.

Determination of pH *of the contents of the intestinal tract.* Determinations were made on (1) fully fed chicks, (2) chicks that had been starved for 18 hr. previously, and (3) chicks that had been fed and given 1 g. alkali, and (4) chicks that had been starved and given 1 g. alkali. The chicks were sacrificed immediately or 1 hr. after the administration of alkali. The crop, gizzard, duodenum, the first 6 in. of ileum and both caeca were removed, opened and placed in 2 oz. bottles and shaken up with 5 ml. distilled water after which they were allowed to stand for 15 min., when the tissues were removed and determinations made on a Cambridge pH meter.

RESULTS

(1) *The production of infections in chickens*

Experiment 1. Three groups, of thirty 1-week-old chickens each, were infected with 1 ml. of a caecal filtrate obtained from ten 3-week-old chickens which were sacrificed 10 days after they had received 1000 embryonated *Heterakis* ova. The groups were treated as follows: group 1—the chickens received food to time of infection; group 2—the chickens were starved for 18 hr. before infection; group 3—the chicks were starved for 18 hr. and then received 1 g. alkali immediately before infection. Ten days later all chickens were sacrificed, and the incidence of caecal and liver lesions was noted (Table 1). A second experiment was carried out on the same lines.

Table 1. *The effect of administering histomonad suspension to fed, starved and starved + alkali-treated chickens*

Group	No. of chickens in group	Treatment of chickens before infection	No. of chickens with caecal lesions	No. of chickens with liver lesions	Total no. of chickens showing both caecal and liver lesions
1	30	On feed	0	0	0
2	30	Starved	5	5	5
3	30	Starved + alkali	25	25	25

Experiment 2. The infective material was also obtained from ten 3-week-old chickens that had received 1000 embryonated *Heterakis* ova and which were sacrificed 10 days later. The incidence of lesions is given in Table 2.

Table 2. *The effect of administering histomonad suspension to fed, starved and starved + alkali-treated chickens*

Group	No. of chickens in group	Treatment of chickens before infection	No. of chickens with caecal lesions	No. of chickens with liver lesions	Total no. of chickens showing both caecal and liver lesions
1	31	On feed	0	0	0
2	31	Starved	3	3	3
3	28	Starved + alkali	28	28	28

Both Expts. 1 and 2 indicate that it is possible to obtain a high incidence of caecal and liver lesions in starved chickens that have received alkali before infection. Starving without alkali permits a low incidence of 'takes', whereas attempts to infect feeding chickens failed. The effect of giving alkali to feeding chickens was considered in the next experiment.

Table 3. *The incidence of lesions produced by administering histomonad suspension to fed and starved chickens, both of which had received* 1 *g. alkali before infection*

Group	No. of chickens in group	Treatment of chickens before infection	No. of chickens with caecal lesions	No. of chickens with liver lesions	Total no. of chickens showing both caecal and liver lesions
1	33	On feed + alkali	7	3	3
2	57	Starved + alkali	55	39	39

Experiment 3. Two groups of thirty-three and fifty-seven 1-week-old chicks, respectively, were each infected with 1 ml. of a histomonad suspension obtained from twelve 3-week-old chickens that had received 1000 embryonated *Heterakis* ova 10 days before they were killed. The two groups of chickens had received the following treatment before they were infected: group 1 was not starved, but received 1 g. alkali just prior to infection; group 2 was starved for 18 hr. and received 1 g. alkali before infection. The chickens were sacrificed 10 days after receiving the infective material. The incidence of lesions is shown in Table 3. Fewer patent infections occurred in unstarved chickens which were given alkali than in starved chickens also given alkali. On the other hand, the administration of alkali produced an incidence of lesions in caeca and livers of unstarved chickens very similar to that observed in starved chickens not receiving alkali (Expts. 1 and 2).

(2) *The development and fate of infections produced by histomonad suspensions*

Experiment 4. This experiment was undertaken to determine the time of the first appearance of lesions in chickens infected in the same way as in the preceding experiments. The histomonad suspension was obtained from ten 3-week-old chickens which were killed 15 days after they had been infected with 600 *Heterakis* ova. Three groups of sixteen 1-week-old chickens each were treated as follows: group 1—in which the chickens were on feed when infected; group 2—in which the chickens were starved for 18 hr. before infection; and group 3—in which they were starved for 18 hr. and then given 1 g. alkali immediately before infection. Ten chicks from each group were sacrificed on the seventh day after infection, and the remainder on the fourteenth day. The incidence of caecal and liver lesions is shown in Table 4. The incidence of lesions in chickens killed on the seventh day is much the same as that obtained in chickens in the previous experiments. Lesions were regressing in chickens of groups 2 and 3 which were sacrificed on the fourteenth day of the infection.

Table 4. *The fate of infections resulting from the administration of histomonad suspension to fed, starved and starved + alkali-treated chickens killed on the seventh and fourteenth days of infection*

			Ten chickens killed on seventh day after infection			Six chickens killed on fourteenth day after infection		
Group	No. of chickens	Treatment of chickens before infection	No. with caecal lesions	No. with liver lesions	No. showing both caecal and liver lesions	No. with caecal lesions	No. with liver lesions	No. showing both caecal and liver lesions
1	16	On feed	0	0	0	1	0	0
2	16	Starved	5	5	5	2	0	0
3	16	Starved + alkali	10	10	10	3	1	1

(3) *The attenuation of histomonad suspensions by repeated passage through chicks*

Experiment 5. The above experiments have shown that the administration of alkali to starved chickens is a valuable preliminary to infection in that there is a substantial production of lesions by the seventh day after infection. As this technique may prove of value in the rapid screening of chemical compounds it was decided to undertake a series of passages from chick to chick to determine the number that could be made before the infective material become attenuated. An attempt at standardizing the method was made by weighing the infected caecal material and adding to it, as diluent, twice its weight of Ringer's solution. As before, caecal material was ground up in a mortar with fine sand, together with the Ringer's solution. The suspension so formed was then squeezed through a single layer of cheese cloth. One ml. of the filtrate was then administered to each chicken *per os* following the 1 g. alkali. The infective material was first obtained from the

caeca of chickens that had been infected with 500 *Heterakis* ova 14 days previously. There was no marked attenuation of the infective material over fourteen passages. No lesions were produced by the fifteenth passage. Variable results have been obtained in subsequent series of passages. As far as attenuation of the infective material is concerned there seemed to be little difference in one series of passages between this method and other methods, not involving the use of *Heterakis* ova for infecting chickens with *H. meleagridis*. In other cases, the suspension became attenuated after only six passages.

(4) *Comparison between infections produced by histomonad suspension and embryonated* Heterakis *ova*

Experiment 6. In this experiment a comparison was made between two groups of 9-day-old White Leghorn chickens treated as follows. Group 1, in which forty-nine chickens were starved for 18 hr. and were then given 1 g. alkali mixture immediately before the administration of histomonad suspension. The suspension was derived from 3-week-old Barred Rock chickens which had been infected 14 days previously with *Heterakis* ova. Thirty-five grams of infected caecal material were ground up with 70 ml. Ringer's solution and 1 ml. of the filtered suspension was given to each chicken. Group 2 was made up of fifty-one chickens which were infected with 1000 *Heterakis* ova. Ten or thirteen chickens from each group were sacrificed on the seventh and fourteenth days after infection. A further eleven chicks from group 1 and thirteen from group 2 were killed on the twenty-first day after infection. Examinations *post-mortem* were made and the progress of the infections in the two groups (Table 5) was compared. The caeca of chickens sacrificed at twenty-one days showed regressive infections. An analysis of the findings *post-mortem* obtained in this group is given in Table 6.

Table 5. *A comparison of the development of lesions in two groups of chickens infected by* (1) *histomonad suspension, and* (2) Heterakis *ova*

Method of infection	Days after infection	No. of chicks sacrificed	No. of chicks showing lesions in caeca*	No. of chicks showing lesions in liver	No. of chicks showing caecal and liver lesions
Histomonad suspension	7	10	10	10	10
	14	10	10	5	5
	21	11	4	0	0
Heterakis ova	7	10	0	0	0
	14	10	10	6	6
	21	13	9	4	4

* Lesions consisted of enlargement of the caeca, thickening of the caecal wall and presence of caecal cores.

In addition to the chickens already mentioned, two more chickens from each group were killed each day from the fourth to the eleventh day and on the fourteenth day after receiving the infective materials the caeca and livers were examined for lesions and an analysis of the findings is given in Table 7.

Table 6. *A comparison of the* post-mortem *findings in histomonad suspension and* Heterakis *ova-infected groups 21 days after infection*

Source of infection	Caecal lesions: Enlargement and cores in both caeca	Caecal lesions: Enlargement and core in one caecum	Caecal lesions: Small amount of necrotic material only	Liver lesions
Histomonad suspension	0 (11)*	1 (11)	3 (11)	0 (11)
Heterakis ova	6 (13)	3 (13)	0 (13)	4 (13)

* Figures in brackets = number of chickens in group.

Table 7. *Comparison of the courses of infections resulting from the administration of* (1) *histomonad suspension, and* (2) Heterakis *ova*

Days after infection	Histomonad suspension (group 1): Condition of caeca	Histomonad suspension (group 1): *Histomonas* in caecal smears	Histomonad suspension (group 1): Condition of liver	*Heterakis* ova (group 2): Condition of caeca	*Heterakis* ova (group 2): *Histomonas* in caecal smears	*Heterakis* ova (group 2): Condition of liver
4	Some thickening of caecal wall with a small amount of necrotic material	+	Normal	Normal	–	Normal
5	Thickened and inflamed walls, hard core of necrotic material present	+	Normal	Few pin-point haemorrhages and contents fluid	+	Normal
6	Very advanced caecal infection	+	Few small lesions	Few pin-point haemorrhages and contents watery	+	Normal
7	Very advanced caecal infection	+	Lesions covering whole of liver	Caeca with inflamed walls. Blood-mucous exudate	+	Normal
8	Advanced caecal infection	+	Lesions still present	As seventh day but condition a little more acute	+	Normal
9	Caeca not as enlarged as on seventh and eighth days. Caecal core still present	+	Few lesions only	Severe infection but no hard core present	+	Normal
10	As ninth day	+	A few necrotic areas on liver only	As ninth day, but hard necrotic core formed	+	Few inflammatory patches
11	As ninth day	+	A few small necrotic spots only	Very advanced caecal infection	+	Advanced lesions
14	Caeca more normal in size and the necrotic core becoming dry and and contracted	+ (less numerous)	Minute lesions only	Advanced caecal infection	+ (less numerous)	Lesions not as numerous as on eleventh day and fewer large lesions present

The results of this experiment indicate that the infections produced by histomonad suspensions proceed more rapidly than those produced by the administration of *Heterakis* ova. In the suspension infection both caeca and livers were heavily involved by the seventh day. Heavy involvement of these organs did not occur until the eleventh day in infections induced by *Heterakis* ova.

(5) *Histomonad suspensions derived from the caeca and livers of turkeys and the production of infections in chickens*

Experiment 7. A single experiment was performed, using the caeca and liver of a turkey that had been killed sixteen days after receiving 500 *Heterakis* ova *per os*. The technique was slightly modified as 16 g. macerated caecal material were ground up with 42 ml. 0·85 % saline and 28 g. macerated liver were ground up with 56 ml. 0·85 % saline. Both suspensions were filtered through cheesecloth. Group 1 was made up of thirty 1-week-old White Leghorn chickens. These chickens were starved for 18 hr. before receiving 1 g. alkali mixture. Fifteen of them received 1 ml. histomonad suspension from caeca, and fifteen 1 ml. infected liver suspension. The thirty chickens composing group 2 were treated in the same way. The chickens of group 1 were maintained on wire floors, and those of group 2 on sawdust litter. This difference in maintenance of the two groups was adopted to determine whether chickens picking up litter might prove to be less easily infected with *Histomonas* than those in contact with food only. The chickens of both groups were killed on the seventh day and the caeca and livers were examined for lesions. The results are given in Table 8.

Table 8. *The incidence of lesions in chickens infected with histomonad suspension derived from caeca and livers*

Maintenance of chickens	No. of chickens in group	Origin of suspension administered	No. of chickens with caecal lesions	No. of chickens with caecal and liver lesions
Wire floor	15	Caeca	15	12
	15	Liver	8	1
Sawdust litter	15	Caeca	14	12
	15	Liver	3	1

There was little difference in the development and incidence of lesions in the two groups receiving caecal histomonad suspension irrespective of whether they were on wire or litter. Further experiments are to be carried out along these lines and will be reported later. There was only a moderate incidence of lesions in the chickens infected with histomonad suspensions derived from the liver.

(6) *The survival of histomonads in suspension exposed to different temperatures for different lengths of time*

Experiment 8. In view of the possible value of the caecal filtrate method in infecting groups of chicks with comparable numbers of Protozoa it was decided to test the longevity of the histomonads under different temperatures and for

different lengths of time. The suspension used in the first series of tests was obtained from the ten caeca of five 2-week-old chicks which had been infected with a suspension derived from infected caeca. Thirty grams of infected caecal material were ground up with 80 ml. Ringer's solution. The suspension was filtered and 1 ml. was administered to each of twenty day-old White Leghorn chickens, which had been starved for 18 hr. and which had received 1 ml. alkali immediately before infection. The remainder of the suspension was divided into three parts, one of which was stored for 2 hr. at 16° C., another at 37° C. and the third at 4·4° C. After treatment, 1 ml. of the suspension was administered to each starved chicken. All chickens received alkali immediately before the infective suspension was administered, and were sacrificed 7 days after they were infected. The results are shown in Table 9.

Table 9. *The effect of storage at different temperatures on histomonad suspensions*

Temperature (° C.)	Time stored (hr.)	No. of chickens receiving filtrate	No. of chickens with caecal lesions	No. of chickens with caecal and liver lesions
37	1	10	6	4
	2	10	5	4
16	1	10	7	5
	2	10	5	4
4·4	1	10	5	4
	2	10	0	0
Filtrate administered immediately	0	20	16	13

A second test was made in which the infective suspension was derived from the caeca of twenty chickens infected with caecal filtrate and killed 7 days later. Otherwise, the method used was similar to that used above. The filtrate was divided into three parts: one part was administered immediately, the second part was stored at 37° C. and the third at 20° C. The filtrates were exposed to these temperatures for 2, 4 and 6 hr. Portions of the filtrate were drawn off at these intervals and administered to starved chickens which had received alkali before the administration of the infective caecal suspension. These chickens were sacrificed on the seventh day of the infection, and the incidence of lesions is summarized in Table 10. Histomonad suspension gives the highest proportion of infections when administered immediately after its preparation. Some infections can be obtained with material maintained at room temperature or at 37° C. for several hours after its preparation. The limiting factors at 37° C. are connected with bacterial overgrowth within the suspensions. At low temperatures, e.g. 4·4° C., no infections were produced by suspension after 2 hr. exposure.

(7) *The* pH *of the alimentary tract of chickens as influenced by feeding, starvation and administration of alkali*

As the effect of different treatments on the pH of the contents of the alimentary tract is being considered more critically and fully in a further communication, only a brief summary of the findings is given in Table 11. On the whole, these

results fall within the ranges of pH observed by other investigators (Farner, 1942, 1943) in birds on normal feed.

When the values given in Table 11 are considered in the light of the results obtained in the infection experiments there appears to be a relationship between the extent to which chickens become infected and the pH of the gizzard at the time of infection. There is an increase in incidence of caecal and hepatic lesions when the contents of the gizzard are alkaline or approach alkalinity at the time of infection, and further work may show that a mechanism similar to that described by Smith (1954) for *Salmonella gallinarum* may also operate in the regulation of *Histomonas* infections.

Table 10. *The effect of storage of histomonad suspension at different temperatures*

Temperature (° C.)	Time stored (hr.)	No. of chickens receiving filtrate	No. of chickens with caecal lesions	No. of chickens with caecal and liver lesions
37	2	10	7	7
	4	10	6	5
	6	10	8	7
20	2	10	7	7
	4	10	4	4
	6	10	4	3
Filtrate administered immediately	0	10	9	8

Table 11. *Ranges of* pH *of contents of the gizzard, duodenum, ileum and caeca of groups of three to five* 1*-week-old chickens receiving different treatments up to time of killing*

Part of alimentary tract	Treatment of chickens at time of pH determinations: On feed	On feed + 1 g. alkali	Starved	Starved + 1 g. alkali
	Ranges of pH in chickens			
Crop	4·1–6·2	6·6–8·3	6·2–7·3	7·5–7·9
Gizzard	2·9–3·3	6·2–6·5	6·3–7·0	7·3–7·6
Duodenum	5·1–6·2	6·2–6·8	6·9–7·1	7·2–7·3
Ileum	5·9–6·1	—	6·0	6·8–6·9*
Caecum	5·8–6·2	—	6·2–6·5	6·5–6·8*

Determinations were made within 15 min. of the chickens receiving alkali except in those indicated (*) which were made 1 hr. after receiving alkali.

DISCUSSION

Attempts to obtain infections by histomonad suspensions derived from infected chickens have always failed when they have been administered to chicks on feed. When the chickens are deprived of food for a period preceding the administration of suspension a small proportion of them become infected. When the chickens are deprived of food and, in addition, are given a small quantity of alkali immediately

before receiving the suspension *per os* a high proportion, if not all, of them develop typical lesions in the caeca and, in many instances, in the liver as well. A study of the pH of the alimentary tract shows that an acid environment (a range of pH of 2·9–3·3) exists in the gizzard of fed chickens owing to the copious secretion of gastric juice that occurs during the feeding process, whereas the range of pH in the crop varies between 4·1 and 6·2 in the same chickens. When the chickens are on feed but have received alkali before the suspension is administered the contents of the gizzard give a pH varying from 6·2 to 6·5 and a small number of chickens develop lesions in the caeca and livers. The gizzard contents of starved chicks give a range of 6·3–7·0, and the incidence of infections obtained is comparable to that of chickens receiving feed and alkali. When chickens are deprived of food and are given alkali the pH rises to give a range of 7·3–7·6 and administered histomonad suspension produces a high incidence of lesions. These findings could be explained by assuming that the upper intestine, and particularly the gizzard, of feeding chickens provides a barrier of low pH which exerts a lethal effect on histomonads which might otherwise enter the lower intestine. The infective suspension passes through the empty gizzards of starved chicks more quickly than it does through full gizzards of feeding chickens where the food is retained throughout the mixing and grinding process before it is released into the duodenum; in this case the histomonads in the gizzard will be exposed to a low pH for a considerably longer time. This tentative interpretation of the results conforms to that of Smith (1954) in his studies with *S. gallinarum*. The passage of viable histomonads into the duodenum may also depend upon the consistency of the food, as well as its influence on the pH of the gizzard contents. These aspects of the problem are now being considered. In this connexion, the technique of histomonad suspension-induced infections may provide a useful means of testing the anti-protozoal activity of chemical compounds. By preparing the suspensions in the manner described, it is possible to use uniform suspensions of histomonads for infecting a number of chickens at the same time. Infections with *Heterakis* ova may also give uniform infections provided that all ova carry histomonads and in the same numbers. A histomonad suspension administered to starved and alkali-treated chickens gives the highest incidence of lesions on or about the seventh day (Expt. 4) and there is a regression of lesions by the fourteenth day (Expt. 6). A *Heterakis* ova-induced infection gives a preponderance of lesions about 4 days later, i.e. by the eleventh day of the infection (Expt. 6). *Heterakis* ova will produce histomonad infections in chickens that are on feed at the time of the administration of ova since they protect the Protozoa from the acid condition of the gizzard. Since infections are produced in feeding chickens by administering *Heterakis* ova, and not by suspension, it may be assumed that *Heterakis* ova are the normal vehicles of natural infections. So far it has proved impossible to maintain viable caecal suspensions over long periods. Should it eventually prove possible to preserve the suspensions the value of the method would be much enhanced since successive groups of chickens could be infected with suspensions of the same origin until such time as they become attenuated (Expt. 5).

SUMMARY

1. Suspensions made from infected caecal material, derived from chickens which had been artificially infected with *Histomonas meleagridis* by the administration of embryonated *Heterakis* ova, can be used to transmit the organisms to other chickens when administered *per os*.

2. Infections with histomonad suspensions can only be obtained in chickens that have been starved, or in chickens that are feeding but have received an alkali mixture immediately before the suspension is administered. The highest incidence of infections is obtained by administering suspensions to starved chickens that have received 1 g. alkali mixture beforehand.

3. Successful infection appears to depend upon the pH of the gizzard and, possibly, of the upper intestine. The incidence of lesions produced by feeding suspension is increased as the contents of the gizzard become alkaline. It is also suggested that the consistency of the food may play a part, as coarse food, along with the infective material, is retained in the gizzard until it is completely ground up and mixed with the gastric juice.

4. In one series of passages the suspension became attenuated after fourteen passages through chickens. In other cases suspensions became attenuated after only six passages through chickens.

5. The courses of infections produced by histomonad suspensions and *Heterakis* ova are compared.

6. The advantages of the method of using histomonad suspensions are discussed in their relation to the screening of chemotherapeutical agents.

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STUDIES ON *SCHISTOSOMA INCOGNITUM* CHANDLER, 1926

I. ON THE SYNONYMY AND MORPHOLOGY OF THE BLOOD-FLUKE

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(With 5 Figures in the Text)

While human schistosomiasis is hardly a problem in India, schistosomiasis in domestic animals is a problem of considerable importance. Blood-flukes infect almost all the species of Indian domestic animals, and often seriously undermine their health and usefulness. Seven species of schistosomes are known to occur in Indian mammals, viz. *Schistosoma indicum* Montgomery (1906*a*), *S. spindalis* Montgomery (1906*b*), *S. incognitum* Chandler (1926), *S. nasalis* Rao (1933), *Bivitellobilharzia nairi* (Mudaliar & Ramanujachari, 1945) Dutt & Srivastava (1955), *Orientobilharzia dattai* (Dutt & Srivastava, 1952) Dutt & Srivastava (1955), and *Orientobilharzia bomfordi* (Montgomery, 1906*b*) Dutt & Srivastava (1955). The reported occurrence of *Schistosoma bovis* (Sonsino, 1876) Blanchard (1895) in India needs confirmation.

In the course of a survey of the incidence of helminth parasites in pigs in the Bareilly district, *S. incognitum* was found to be quite common. This blood-fluke has so far been known to occur in India only. Its gross morphology and occurrence in pigs and dogs at different places in the country have been described by several workers (Rao & Ayyar, 1933; Rao, 1935, 1937; Bhalerao, 1934, and Srivastava, 1942). But very little work on its life history has been done beyond a brief description of its miracidium by Rao (1937), and the bare mention by Khaw (1947) about its molluscan intermediate host. Khaw did not describe the cercaria or mention its characteristic features but merely remarked, 'Two forms of furcocercous cercariae, microscopically resembling those of man, proved on passage through rabbits to belong to two species of schistosomes of cattle—*S. indicum* Montgomery (1906) and *S. suis* Rao and Ayyar (1933). Though both kinds of snails were plentiful in the two regions, the cercaria of the former was detected in six per thousand of *Indoplanorbis exustus* in Bihar only, and that of the latter in thirteen per thousand of *Lymnaea* (*luteola*) f. *succinea* or *ovalis* in Madras, Chittar district only. The adults have been recorded from cattle from the two provinces by Malkani from Bihar and Rao and Ayyar from Madras.' Khaw's statement that the adult *Schistosoma incognitum* had been recorded from cattle in Madras by Rao & Ayyar is incorrect, for in their paper there is no mention of cattle being the definitive host of the parasite. The joint authors had reported its occurrence in pigs and dogs only.

In view of the common occurrence of the blood-fluke in pigs and dogs and its pathogenic significance as indicated by Rao (1937), a detailed study of the different

aspects of its biology and host-parasite relationship has been carried out. This formed the subject of two papers read by the authors in the Section of Medical and Veterinary sciences of the 41st Session of the Indian Science Congress Association, 1954 (Sinha & Srivastava, 1954). For purpose of publication, the work is presented in three parts: Part I—deals with the synonymy and morphology; Part II—the life history; and Part III—the bionomical studies on the free-living stages of the parasite.

For these investigations, large numbers of male and female specimens of *S. incognitum* were obtained initially from the entrails of pigs slaughtered in the pork market. Infected livers always yielded a large number of specimens. A regular supply of the adult specimens of both sexes and of mature eggs was made available throughout from experimentally infected clean hosts maintained in the laboratory. Various species of animals, such as pig, dog, cat, sheep, goat, cattle, rabbit, and guinea-pig, etc., were infected separately with the cercaria obtained from naturally and experimentally infected snails, and they yielded a good number of specimens. A heavily infected sow was also maintained at the laboratory as a donor of parasite material. Specimens of the cercaria were collected from naturally and experimentally infected intermediate hosts for the study of its morphology and biology. The identity of the larva from naturally infected snails was made sure by infecting clean laboratory animals. The definitive hosts used were mostly bred and maintained in the laboratory under parasite-controlled conditions and such of them as were obtained from outside were determined to be free from infection before they were used.

The authors are grateful to Dr S. Datta, till recently Director of the Indian Veterinary Research Institute, for his keen interest in these studies.

SYNONYMY OF *SCHISTOSOMA INCOGNITUM* CHANDLER, 1926, AND *S. SUIS* RAO & AYYAR, 1933

Owing to the averseness of some people in India to submitting individual stool samples for examination Chandler frequently examined freshly passed stools collected from the fields used as defaecation areas in villages, for collection of data on helminthic infections. One of the 120 such samples collected from a village in Bengal was found to contain, besides hookworm ova, schistosome eggs quite different from those of the previously described species. The field from which the samples were collected was used as a defaecation ground by a low-caste population —Dome—who kept pigs. Pigs from the village also frequently visited the area. From the nature of the stool Chandler thought there was no reasonable doubt but that it was a human stool which contained the eggs. Subsequently, a second case of the same infection was detected in a sample of stool collected in the same manner from the vicinity of another village in Bengal. As in the previous case, pigs were common in the locality from which the stool was collected. He gave accurate description and measurement of the eggs and pointed out their distinguishing features and appended a key to the identification of species of *Schistosoma* on the basis of their eggs. Though nothing was known of the adult worms, the

characteristic features of the eggs were considered sufficient for the creation of a new species—*S. incognitum*.

In 1933, Rao & Ayyar described a new species—*S. suis*—parasitic in pigs in Madras. Subsequently, it was found to occur in dogs also, in different parts of the country. In the discussion on the systematic position of the new species, the joint authors recognized that the ova described by Chandler (1926) resembled in all respects those of *S. suis* and even suggested the possibility that the faeces in which Chandler had found the ova might have been from the pig and not from human beings. Nevertheless, they gave it a new name because the adult worms and their host had been found, and tentatively placed *S. incognitum* as a synonym of *S. suis*, regarding the former and older name as *nomen nudum*.

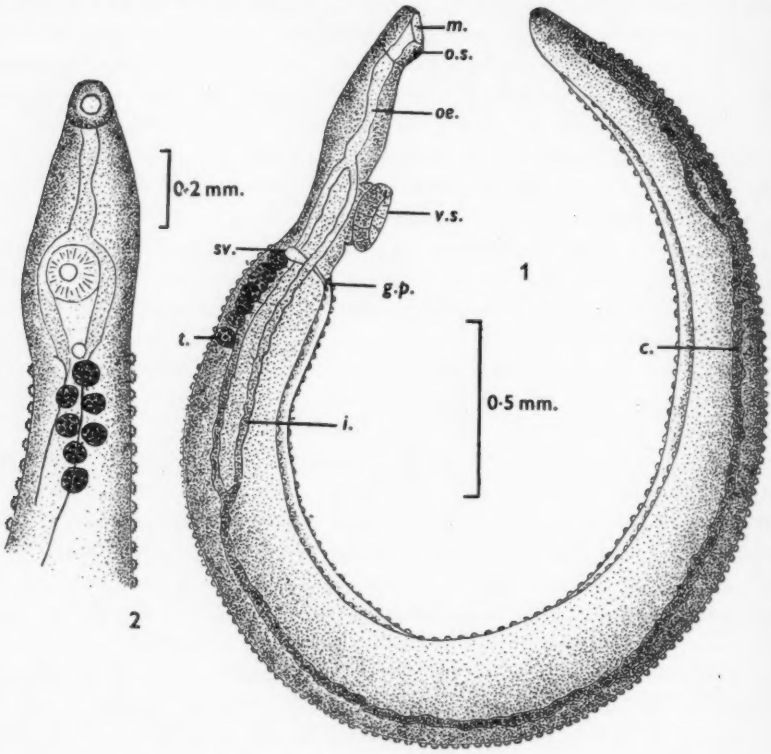
Article 25 (*a*) and (*b*) of the International Code of Zoological Nomenclature provides that: 'The valid name of a genus or species can only be that under which it was first designated on the condition: (*a*) that (prior to January 1, 1931) this name was published and accompanied by an indication, or a definition, or a description; and (*b*) that the author has applied the principles of binary nomenclature.' Further, Article 27 (*b*) provides that the Law of Priority obtains and consequently the oldest available name is retained when any stage in the life history is named before the adult. According to the provision of these Articles the name *S. incognitum* Chandler, 1926, becomes valid and *S. suis* Rao & Ayyar, 1933, its synonym. The question whether the faeces from which Chandler got the eggs was from a pig or human being is irrelevant to the determination of the synonymity of the two names given to the parasite. It is well known that, within reasonable limits of variation, the eggs of different species of schistosomes have a definite and characteristic shape which is of recognized taxonomic value in separating them. Most of the species of blood-flukes have no marked single final host specificity, and the absence of a definite information as to the host of *S. incognitum* does not at all detract from the validity of the name given by Chandler. Bhalerao (1937) also held the view that if *S. incognitum* and *S. suis* referred to the same blood-fluke then the latter fell into synonymy. Bhalerao (1934) described as *S. japonicum* specimens of a schistosome collected from the intestine of pigs in Calcutta, and sent to him by Dr P. A. Maplestone. Later on he (1948) referred them to *S. incognitum*.

MORPHOLOGY OF *SCHISTOSOMA INCOGNITUM*

The male of *S. incognitum* is very stout and whitish in colour. The female is filiform and its posterior two-thirds are slightly darker and stouter than the pale, translucent, thin and delicate anterior-third. The male worms can contract to a considerable extent and assume different shapes. The anterior portion sometimes becomes elongated, cylindrical and arches backwards with the ventral sucker prominently pushed out and constantly changing its form. The posterior portion of the body then becomes short, broad and bent inwards. Sometimes the marginal folds open out and show wavy movements along the long axis. At other times the anterior end becomes short, almost flattened like the posterior portion of the body withdrawing the acetabulum inside the hollow formed on the ventral aspect. The

oral sucker is also contracted and directed ventrally. The female worms have very poor power of contraction. Sometimes they bend their body when the ventral sucker protrudes and changes its shape. Worms in copula are not uncommon.

The male. To study the morphology of the male worms, specimens were collected from the liver and portal veins of several animals belonging to each of the different



Figs. 1, 2. *Schistosoma incognitum.*

Fig. 1. Adult male lateral view.

Fig. 2. The anterior end of the male, ventral view.

species of its hosts. The measurements were taken of permanent preparations of adult worms. The length and breadth were measured of twenty-six specimens from three pigs, eighteen specimens from two dogs, thirty-three specimens from three rabbits, twenty-three specimens from three guinea-pigs, twenty specimens from two white rats, fifteen specimens from two white mice, five specimens from two goats and twelve specimens from two cow calves. The measurements of different organs given in the account cover all the worms measured for their length and breadth except those from the rats and mice.

The body of the male worm (Fig. 1) is divided into two distinct parts, a short cylindrical anterior portion and a longer flat posterior portion with its sides folded on the ventral aspect to form the gynaecophoric canal. The narrower anterior

portion after a slight constriction abruptly widens out to form the posterior portion. The adult male worm is 2·43–8·86 mm. (average 5·8 mm.) long and 0·1–0·457 mm. (average 0·296 mm.) in maximum width across the middle of the body. The anterior portion of the body is 0·29–0·97 mm. (average 0·74 mm.) long. The width of the anterior portion at the level of the constriction behind the ventral sucker is 0·07–0·2 mm. (average 0·13 mm.). The posterior portion of the body gradually narrows down to the hinder end which is rounded. The maximum width of the posterior portion is 0·1–0·43 mm. (average 0·29 mm.). The measurements of both male and female specimens obtained from different hosts are given in Table 1.

Table 1. *Measurements (in millimetres) of the adult male and female specimens of* Schistosoma incognitum *obtained from different hosts*

	Males		Females	
Host	Length	Breadth	Length	Breadth
Pig	5·14–7·57 (av. 6·71)	0·214–0·457 (av. 0·29)	4·3–7·11 (av. 5·93)	0·1–0·112 (av. 0·11)
Dog	6·4–8·3 (av. 7·7)	0·33–0·43 (av. 0·4)	4·8–7·57 (av. 6·5)	0·1–0·112 (av. 0·116)
Rabbit	5·0–8·86 (av. 6·33)	0·157–0·43 (av. 0·314)	4·5–7·11 (av. 5·85)	0·086–0·14 (av. 0·112)
Guinea-pig	2·64–6·80 (av. 5·42)	0·13–0·414 (av. 0·294)	2·57–6·7 (av. 5·11)	0·05–0·114 (av. 0·1)
White rat	3·57–4·7 (av. 4·1)	0·13–0·314 (av. 0·23)	2·7–3·0 (av. 2·85)	0·0715–0·13 (av. 0·081)
White mouse	2·43–4·7 av. 4·06)	0·1–0·286 (av. 0·23)	3·0–3·14 (av. 3·55)	0·0715–0·13 (av. 0·095)
Goat	5·3–6·7 (av. 5·9)	0·314–0·37 (av. 0·351)	5·7–7·14 (av. 6·43)	0·114–0·143 (av. 0·132)
Cow calf	3·14–7·14 (av. 5·137)	0·114–0·357 (av. 0·277)	(Only a few female specimens were obtained from cow calves, but they were all either broken or contracted and unsuitable for accurate measurements.)	

The cuticle of the posterior portion of the body bears minute spines ventrally and is embossed dorsally with scattered tubercles. Each tubercle bears about fourteen to twenty spines which are very weak, and in preserved specimens many of them are lost in handling. The suckers are also covered with minute spines. The worms when kept for a long period in normal saline solution show partial sloughing of the cuticle or formation of vesicles which partially obliterate the tubercles. The anterior portion of the body does not possess spines or tubercles. The anterior portion of body bears the two suckers. The oral sucker at the anterior end is obliquely set and is smaller than the ventral sucker. The oral sucker is 0·1–0·2 × 0·08–0·18 mm. (average 0·15 × 0·12 mm.). Its dorsal lip is longer than the ventral, the former being the anterior-most extremity of the body. The rounded

rim of the sucker is thick and muscular. The ventral sucker is round, pedunculated and 0·085–0·26 mm. (average 0·16 mm.) in diameter.

In a cross-section of the posterior body is seen a tuberculate cuticle as the outermost layer. The subcuticular layer consists of a thin layer of circular muscle fibres and a thick layer of ectodermal cells with prominent nuclei interspersed with the cut ends of the bundles of longitudinal muscle fibres. The parenchyma consists of thin-walled, loosely packed areolar connective tissue with nucleated parenchymatous cells. The cuticle of the ventral aspect shows minute spines and the subcuticular layer of circular muscle fibres is comparatively thicker.

The digestive system consists of the mouth, an oesophagus and two intestinal caeca. The mouth is situated subterminally and the funnel-like oral cavity leads into an oesophagus varying in length from 0·16 to 0·36 mm. (average 0·257 mm.). Just in front of the ventral sucker the oesophagus bifurcates into two intestinal caeca, which unite at a distance from the anterior end varying from 1 to 2·14 mm. (average 1·49 mm.). The common caecum then runs medially to about 0·1–0·27 mm. (average 1·54 mm.) from the posterior end. It bifurcates at one or two places and then the branches unite again after short distances. Very often the gut of the living worms contains partly digested blood which makes them quite conspicuous.

The excretory system consists of a pair of tubes running parallel to the long axis of the body. The two tubes unite at only a short distance from the posterior end and form an elongated excretory vesicle which opens at the hinder end of the body. In cross-section the excretory tubes are found on the outer sides of the caeca.

The testes are more or less spherical, slightly dark in colour, and vary from two to seven in number. On an examination of one hundred and seventeen specimens from different hosts forty had seven testes, thirty-eight had six, twenty-eight had five, eight had four, two had three and one had two testes. When viewed laterally the glands appear to be in one line, and except the first and the last they partly overlap one another. They appear to be situated at different depths. In a dorso-ventral view they lie in two irregular lines—the first, third and the fifth more or less form one line, and the second, fourth, sixth and the seventh form the second line (Fig. 2). The testes lie dorsally to the two intestinal caeca. The seminal vesicle is an oblong sac in front of the testes and measures 0·026–0·0485 × 0·026–0·0323 mm. (average 0·0387 × 0·026 mm.), opening to the exterior by a canal of almost the same length. The genital pore lies at the beginning of the gynaecophoric canal.

The female. The female worm is usually shorter and much more slender than the male (Fig. 3). The body is cylindrical, tapering gradually towards the ends. The greatest width is across the region near the ovary. The posterior two-thirds occupied by the vitellaria are slightly thicker than the anterior third. The length and breadth were measured of eighteen specimens from three pigs, twenty-six specimens from two dogs, twenty-five from three rabbits, nineteen from three guinea-pigs, four from one white rat, seven from two white mice and seven from two goats. The adult female worm is 2·57–7·57 mm. (average 5·72 mm.) long and 0·05–0·14 mm (average 0·11 mm.) in maximum width.

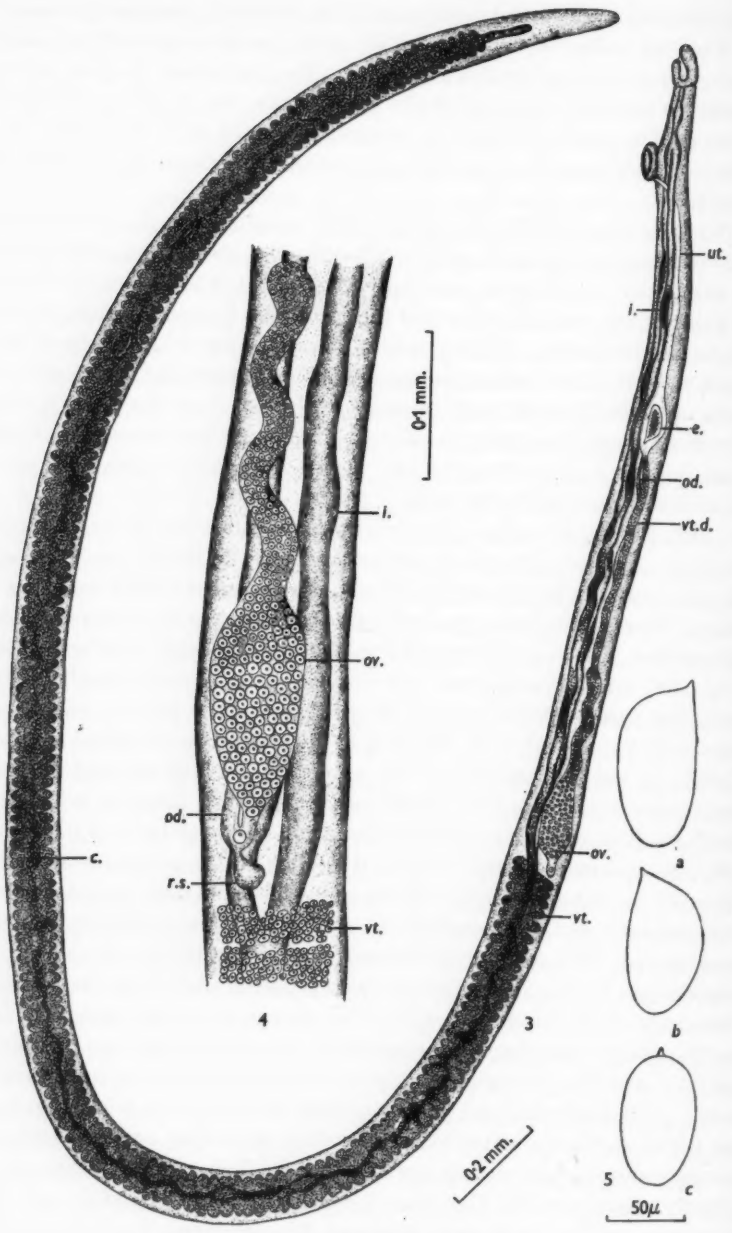
The cuticle of the female worm bears very minute spines on the body and the

suckers. The oral and ventral suckers are very weak as compared to those of the male. The oral sucker is placed obliquely at the anterior end and, unlike that of the male, is larger than the ventral sucker. Its dimensions vary from 0·051 to 0·1 × 0·028 to 0·07 mm. (average 0·078 × 0·05 mm.). Its dorsal lip is larger than the ventral. The ventral sucker is pedunculated and 0·03–0·057 mm. (average 0·05 mm.) in diameter. The musculature is arranged as in the males but is far less developed.

The digestive system in the female is more or less similar to that in the male. The infundibular oral cavity leads posteriorly into the oesophagus, which varies in length from 0·07 to 0·21 mm. (average 0·0986 mm.). The oesophagus bifurcates just in front of the ventral sucker and the caeca pass backwards along the sides of the uterus and the ovary. Slightly behind the ovary where the vitellaria start the caeca unite to form a common caecum which runs medially surrounded by the vitellaria up to 0·028–0·186 mm. (average 0·13 mm.) from the posterior end. In its downward course the common caecum bifurcates a few times. The bifurcated branches run for a short distance only before they unite again. The excretory system is similar to that in the male.

The ovary (Fig. 4) is made up of a solid mass of cells forming the major portion of the organ. Its anterior end is narrow and spirally elongated. The stalk-like anterior end varies in length which accounts for the great variations in the size of the ovary. However, in most specimens the anterior portion is more or less equal to the posterior. The ovary is from 0·3 to 0·47 mm. (average 0·357 mm.) long and 0·028 to 0·085 mm. (average 0·05 mm.) broad. It is placed dorsally to the two intestinal caeca and a little in front of their union. The oviduct arises from the posterior end of the ovary and runs first backwards for a very short distance and then bends forward extending to the ootype. A sac-like seminal receptacle is situated posteriorly to the loop of the oviduct and just anterior to the origin of the vitellaria. The vitellaria occupies about two-thirds of the body length. It is 1·54–5·8 mm. (average 4·7 mm.) long and lies behind the ovary and extends from the union of the intestinal caeca to about 0·028–0·186 mm. (average 0·13 mm.) from the posterior end. The vitellaria consists of follicular glands surrounding the common caecum. The main vitelline duct runs ventrally to the caecum, full of yolk secretions. It joins the oviduct where it swells slightly to form the ootype into which the shell glands also open. The ootype continues anteriorly into the uterus. The uterus runs forward between the intestinal caeca and opens through the genital pore at the posterior margin of the peduncle of the ventral sucker. The uterus is a prominent tube and varies in length from 0·257 to 0·757 mm. (average 0·4 mm.). The uterus was never found to contain more than one egg at a time. The uterine egg resembles the mature egg found in the faeces of the host in shape, but it is slightly smaller in size. The spine is developed as in the mature egg. The size of thirty-five uterine eggs from different female worms varies from 0·086 to 0·114 mm. (average 0·098 mm.) in length and 0·036 to 0·043 mm. (average 0·039 mm.) in breadth. The spine is 0·005–0·0075 mm. long (average 0·0065 mm.).

The mature egg passed out with the faeces of the host is yellowish brown in colour. It is thin shelled and suboval in shape, with one of its sides comparatively



Figs. 3–5. *Schistosoma incognitum.*

Fig. 3. Adult female *Schistosoma incognitum.*

Fig. 4. Portion of the female showing the ovary.

Fig. 5. Mature eggs passed in the faeces: (*a*) and (*b*) lateral view; (*c*) lying on its flat side.

flattened (Fig. 5). The short stout spine is situated asymmetrically at one end more or less in continuation of the flattened side of the egg. The size of the egg varies from 0·097 to 0·148 mm. (average 0·118 mm.) in length and 0·045 to 0·081 mm. (average 0·061 mm.) in breadth. The spine is on an average 0·007 mm. long (variation from 0·005 to 0·013 mm.). From the measurements of 150 specimens collected from the faeces of two representatives of infected pigs, dogs and guinea-pigs, it is seen that the size of the eggs from pigs and dogs has the same range of variation (0·110–0·148 × 0·058–0·081 mm.), but the eggs from the guinea-pigs are much smaller (0·097–0·123 × 0·045–0·061 mm.). The average size of the eggs from pigs and dogs is 0·123 × 0·067 mm., while those from guinea-pigs measure 0·106 × 0·048 mm.

The description of the morphology of *S. incognitum* by Rao & Ayyar (1933) is based on a study of five adult specimens of each sex, and that by Bhalerao (1934) on six poorly preserved male worms. Though the present observations, based on the study of a large number of living and well-preserved specimens from different species of hosts, generally tally with the descriptions given by the previous authors, they have revealed a much wider range of variations in size of the worms (Table 1), their organs and eggs.

The account of *S. incognitum* by the previous workers has been supplemented in respect of several characters of taxonomic importance. Rao & Ayyar (1933) had described only a very short length of the cornua of the excretory vesicle. In these studies the cornua forming the excretory tubes has been traced throughout the length of the body in cross-sections. The number of testes according to the previous authors varies from four to seven, but in the present study they have been found to range from two to seven, the large majority possessing five to seven. The authors' observations on the shape and arrangement of the testes agree with those of Bhalerao. Rao & Ayyar have described the ovary as a pyriform body, but in most of the specimens in our collection it has a posterior oval portion and an anterior spiral elongated structure which varies considerably in length. The account of the connexions of the female genital ducts given by the joint authors is very meagre. They mentioned that 'the vitelline ducts meet the oviduct a little behind its origin'. It has been definitely found that the main vitelline duct is one, and it runs ventrally to the common caecum to join the oviduct near the ootype.

Abbreviations used in figures:

c.	caecum	*ov.*	ovary
e.	eggs	*r.s.*	receptaculum seminis
g.p.	genital pore	*s.v.*	seminal vesicle
i.	intestine	*t.*	testes
m.	mouth	*ut.*	uterus
od.	oviduct	*v.s.*	ventral sucker
oe.	oesophagus	*vt.*	vitellaria
o.s.	oral sucker	*vt.d.*	vitelline duct

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ON *CORRIGIA* (*ORTHORCHIS*) *VITTA* (DUJ. 1845)

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(With 1 Figure in the Text)

INTRODUCTION

Dujardin's original description of *Lyperosomum vitta* was based on the posterior portion of a single trematode found in the intestine of a wood mouse, *Apodemus sylvaticus*, at Rennes. Subsequent to this the parasite was apparently not observed again until the 1920's when the material collected by Elton, Ford & Baker (1931) during their survey of the parasites of small mammals in Bagley Wood was submitted to Baylis. Again, Baylis (1927) found only fragments of worms, these being anterior portions taken from the anterior intestinal region of *Apodemus sylvaticus*. Although large numbers of voles were included in the material obtained from Bagley Wood, none of these yielded any specimens of the parasite. The fragments were found in only six of a total of 692 mice examined, a fact which led the authors of the report to conclude that this parasite was of little significance to the well-being of their hosts. Shortly after this, Baer (1932) reported the true habitat of the worm, and briefly described the whole animal for the first time. Having found posterior fragments in the intestine of two mice during autopsy, and failing to discover the remaining parts in the gut, he examined the associated organs of the alimentary system and was able to establish that the parasites live normally in the interlobary canals of the pancreas. He reported the animals as occurring in two hosts in the neighbourhood of Pinchet, near Geneva, these being *A. sylvaticus* and *Evotomys glareolus helveticus* Mill. Baer's material presented some differences from that seen by Baylis, in particular the eggs being distinctly smaller, while some diversity was also apparent in the proportions of the various organs of the body.

OCCURRENCE NEAR EXETER

Recently, being then unaware of Baer's report, we were able independently to confirm his observations on the habitat occupied by the worm. The junior author (C.E.C.), while examining an *Apodemus*, found two worms entangled in the remains of the pancreas after having disentangled the intestine. Subsequent search of other mice revealed the parasites in the interlobary ducts of the pancreas, where they were readily seen on spreading the pancreas, as Baer remarked, on account of the black or dark chocolate colour of the eggs in the uterus. Baer gave no indication of the frequency of incidence in the Swiss mice, merely remarking that the worms were found in nine *Apodemus* and three *Evotomys*. In all we have examined the following animals: 34 *Apodemus sylvaticus*; 2 *Clethrionomys glareolus* (formerly *Evotomys*); 1 *Microtus agrestis*; 1 *Sorex araneus*. The last three species

yielded negative results. Of the 34 *Apodemus*, 13 were infested, a comparatively high rate of 1 to 2·6.

These small mammals were supplied to us by Mr W. R. Pickering of this department, having been captured in three localities in or near Exeter, as follows:

Area 1	Reed Woods, Exeter	19 mice, 9 infested
Area 2	Bramble patch about 300 yd. east of area 1	9 mice, 1 infested
Area 3	House and garden, Exminster, 5 miles south-east of Exeter	6 mice, 4 infested

In every case the intestine was searched in addition to the pancreas, and in one instance only were fragments found here. It is evident, therefore, that the species is by no means uncommon, and had the pancreas been examined in the animals taken by Elton, Ford & Baker they would have revealed a much higher rate of infestation than the 6 in 692 actually recorded.

Little information is given by Baer as to the number of worms which may occur in a single host. He remarks that eight animals were extracted from a single duct, in which they were lying closely packed side by side. He also figures a section of a duct containing three parasites. The figures obtained by Elton, Ford & Baker cannot of course give any clear indication of actual numbers present. Mr Elton informs us, however (1954), that of the six parasitized mice, four yielded single fragments while two and five pieces, respectively, were found in the other two hosts. The distribution of parasites in our material was as follows:

No. worms per host	No. infested hosts
1	6
2	2
3	1
5	2
6	1
8	1

The case in which eight parasites were present in the one host was interesting in that the worms were at different stages of maturity. Four were well grown and had reached sexual maturity, as evidenced by the abundance of eggs in the uterus. The remainder were small, only some one-third to one-half the adult length, the gonads were immature and no ovulation had occurred. It is possible that superinfection may have occurred, the smaller animals representing a second, new, infection subsequent to the original one. On the other hand, the condition may have arisen from a single heavy infection which was too large to be supported by the host, with the result that a proportion of the worms became retarded and stunted in growth.

Examination of serial sections of an infected pancreas, for the preparation of which we are indebted to Dr R. S. J. Hawes, has enabled us to confirm Baer's observations that the lining of the pancreatic duct shows no obvious pathological changes. Particularly, where two or more parasites are lying side by side the duct becomes grossly distended, but no signs of erosion or inflammation have been detected, and it would seem that the worms do not attack the epithelial lining.

Like Baer, we found that the extraction of whole worms to be a delicate operation, best performed on the freshly killed animal. By cutting an incision in the wall of the duct the worm could then be extracted by a combination of gentle pulling by means of a needle or seeker inserted beneath it and of pressure on the two ends of the worm still lying in the duct. It sometimes happened that pressure of work forced us to store killed mice overnight under refrigeration. The parasites survived this procedure for up to 24 hr., and could be extracted alive. They were then, however, very much more fragile and it was more difficult to remove them intact.

STRUCTURE AND PROPORTIONS

In view of the discrepancies in the recorded measurements of material of the worm we have examined our material carefully, using only mature, egg-laying animals. The measurements are recorded in Table 1, together with the comparable figures obtained by Baylis and Baer respectively. It will be noted that our measurements

Table 1. *Dimensions in* Corrigia vitta (*all figures in* μ)

Organ	No. of measurements	Range of measurements	Average measurements	Comparable figures	
				Baylis 1927	Baer 1932
Anterior sucker					
Diameter	9	210–280	242	300–310	300
Ventral sucker					
Diameter	11	210–300	269	400–450	340
Pharynx					
Length	7	75–120	101	150	130
Diameter	7	100–135	113	170	160
Cirrus sac					
Length	5	105–345	200	300–350	290–330
Diameter	5	60–105	81	100–140	100–160
Testis 1					
Length	10	105–270	197	—	—
Diameter	10	135–360	256	—	—
				300–470	
Testis 2				230–300	
Length	10	105–300	194		—
Diameter	10	135–360	254		—
Ovary					
Length	10	135–210	160	320	—
Diameter	10	165–325	256	250	—
Vitellaria					
Left	6	1690–3930	2810	—	—
Right	6	1500–3250	2190	—	—
Egg	—	—	36·9 × 18·2	45 × 22·5–25	38 × 23

are for the most part distinctly smaller than those recorded by either Baylis or Baer, a fact which may possibly be related to the methods of fixation employed. Our own animals were fixed in Bouin's fluid. The wide diversity in our own material, however, indicates that the proportions are very variable, this possibly being correlated to some extent with the state of the gonads. In the cases of the cirrus

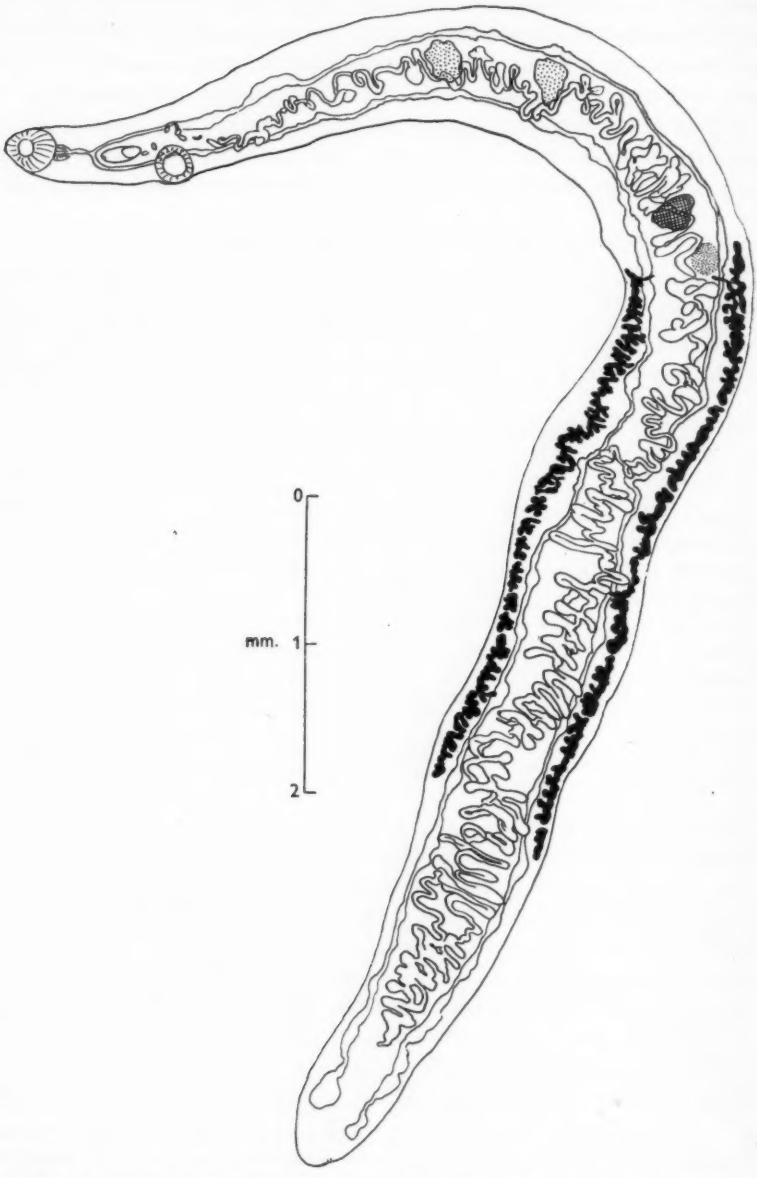

Fig. 1. Whole mount of *Corrigia vitta*. (Bouin's fluid, weak Delafield's haematoxylin.) Camera lucida drawing under 42 mm. obj., × 10 ocular.

sac and the receptaculum, for instance, the proportions depend to a large extent on the degree to which they are distended with spermatozoa.

Certain details require special mention:

(i) Baer drew attention to the disparity between his measurements of the egg and those of Baylis, the former's being distinctly the smaller. Our own figures approximate very closely to those of Baer, and it would seem that these may be regarded as the more normal.

(ii) Both Baylis and Baer describe the testes as ovoid bodies with their long axes transverse to the long axis of the animal, and the ovary as more nearly spherical. In our material we have found these organs all to be variable in shape. Both ovary and testes may be distinctly lobulated (cf. Fig. 1), regularly ovoid to subspherical or not infrequently wedge-shaped in section. Invariably the greatest length is transverse to the long axis of the animal.

(iii) The vitellaria, as may be seen in Fig. 1, and as reported by Baer, are asymmetrically developed. While both commence at a level immediately posterior to the ovary and receptaculum seminis, one invariably extends farther in a posterior-wards direction than the other, usually by between 10 and 20 % of the length of the shorter. In one extreme case the difference was by some 67 %, the lengths of the two vitellaria being 3·45 and 2·06 mm. respectively.

NOMENCLATURE

The genus *Lyperosomum*, of the family Dicrocoeliidae, originally proposed by Looss 1899 for *L. longicauda* (Rudolphi, 1809), was revised by Travassos in 1919 and again in 1944 (Travassos, 1944) when, among others, the genus *Orthorchis* was separated off for the type *Lyperosomum corrigia*. *Orthorchis* was distinguished from *Lyperosomum* by the median alignment of its testes, and the fact that the vitellaria do not extend anterior to the ovary, whereas in *Lyperosomum* the latter extend at least as far forwards as the posterior testis, while the testes themselves are disposed one behind the other on a diagonal line. Moreover, in the latter genus the diverticula of the gut stop well short of the posterior end of the body, whereas in *Orthorchis* they extend very nearly to the hind-end. All our material, together with that described by Baylis and Baer, conforms with Travassos's genus *Orthorchis*, and it must therefore be removed from the genus *Lyperosomum*. But, as Dollfuss (1954) has pointed out, Travassos's genus *Orthorchis* must give priority to *Corrigia* Strom 1940, he having revised *Lyperosomum*, unknown to Travassos, in 1940 and separated off as *Corrigia* those species having the characters of Travassos's *Orthorchis*. The animal with which we are here concerned is therefore *Corrigia* (=*Orthorchis*) *vitta* (Dujardin 1845).

SUMMARY

1. Of thirty-four *Apodemus sylvaticus* examined from in and near Exeter, thirteen were infested with varying numbers of the dicrocoeliid trematode, *Corrigia vitta*.

2. The worms live in the interlobary canals of the pancreas, sometimes singly, but up to eight have been found in a single mouse.

3. The dimensions of the more important organs are stated and compared with the measurements provided by other authors.

4. The taxonomic status of the species is briefly considered.

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A HISTOCHEMICAL APPROACH TO THE STUDY OF HELMINTH MORPHOLOGY

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(With Plate II and 1 Figure in the Text)

The development of histochemical methods has been particularly rapid during recent years, and the appearance of several excellent text-books have made the techniques generally available (Pearse, 1953; Gomori, 1952; Glick, 1949). As many helminths contain organ systems rich in a particular substance or enzyme, it is clearly only a matter of time before histochemical methods will find application in the demonstration of helminth morphology—particularly as studied in whole mount preparations.

Helminthologists are understandingly reluctant to depart from well-known and well-tried methods, commonly involving fixation with corrosive sublimate or other fixatives, followed by staining with some variety of carmine or haematoxylin. These methods, excellent as they often are in the hands of careful workers, are essentially empirical, for no care is taken to preserve particular substances or enzyme systems.

It is the purpose of this paper to put forward several histochemical methods which are readily adapted to helminths, particularly trematodes and pseudophyllidean cestodes, and which, in our opinion, produce results equal to, and often greatly superior to, those obtained by routine methods.

These new methods, when used for observation at a cytochemical level, often need very precise control of factors such as reagent concentration, temperature and pH, but when used for observation at a macroscopic level often require much less rigid conditions. We have attempted, therefore, to reduce these methods to the simplest compatible with efficiency, so that they can be used on the widest possible scale and in laboratories where complex apparatus or rare chemicals may not be readily available.

THEORETICAL CONSIDERATIONS

These methods are mainly based on the fact that the vitelline glands of most trematodes and many pseudophyllidean cestodes are rich in proteins and phenols (Smyth, 1954). These materials are released during egg formation and combine to form a quinone-tanned egg-shell. The formation of quinone-tanned proteins takes place as follows:

$$\text{protein} + o\text{-diphenol} \xrightarrow{\text{polyphenol oxidase}} \text{protein} + \text{quinone}$$

$$= \text{quinone-tanned protein (sclerotin)}$$

—the quinone produced forms cross-links with the protein chains and thus produces a stable resistant protein.

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It is now known that the diphenol constituent is unnecessary, for protein containing tyrosine side-chains when acted on directly by polyphenol oxidase produces sufficient quinone nuclei to tan itself (Hackmann, 1953)—a process we have termed auto-quinone tanning (Smyth, 1954).

Although we are uncertain exactly which of these alternative mechanisms is followed during the formation and hardening of the egg-shell in trematodes, the result from the histochemical point of view will be the same—namely that the cells of the vitellaria should give positive results for (*a*) polyphenol oxidase, (*b*) phenols, and (*c*) proteins; the eggs low down in the uterus (i.e. those freshly formed) should likewise react to the same tests. As the eggs pass up towards the mouth of the uterus they darken in colour and finally become hardened and tanned.

Many of the histochemical tests for these substances are unsuitable for use at a macroscopic level, as they involve complicated techniques or give colour reactions which are either too weak or diffuse to give useful results. Some are excellent for whole mount preparations but useless for sections; with others the reverse is the case.

GENERAL TREATMENT OF MATERIAL

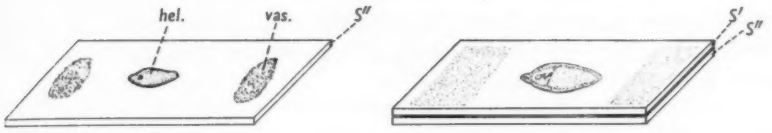
Collection

Although most parasitologists realize that material should be fixed as soon as possible after the death of the host, this procedure is often not carried out. It is particularly important to do so when dealing with techniques based on enzyme distribution where severe post-mortem changes may bring about a diffusion of the enzyme. In the case of parasites of warm-blooded animals, it is not generally appreciated that they may frequently be reactivated by incubating for a short period in saline at the correct temperature (37–40° C. depending on the host). Thus, for example, it is normally our practice when collecting *Fasciola* from infected livers to transport them to the laboratory in Ringer-Locke at 37° C. in a large vacuum flask, and further incubate them in Ringer-Locke or Tyrode at the same temperature for 1–2 hr. Under these conditions, the majority of flukes become active and hold on to the sides of the culture vessel; such specimens are clearly healthy, worthy of fixing and in general produce excellent preparations; whereas those which show little or no activity may be discarded as being in a dying or degenerating condition.

Flattening of specimens

The most widely used method for flattening cestodes and trematodes (at least in the British Isles) is to compress specimens between slides and bind these with cotton or press down with weights. Both these methods suffer from the disadvantage that the amount of pressure is difficult to control, with the result that specimens are not infrequently damaged. A method introduced to this Department by one of us (L. N. J.) and long used in certain Indian Universities does not suffer from this disadvantage and permits the amount of pressure to be controlled within very delicate limits.

The method is illustrated in Text-Fig. 1. The surfaces of the specimen are superficially dried on filter-paper, and it is placed on a slide bearing a dab of vaseline jelly at either end; a second slide is placed on top and pressed down gently until the specimen is sufficiently flattened. When pressure is removed it is found that the adhesive properties of the vaseline are sufficient to maintain the two slides together and they may now be lowered into the fixative. In rare cases (e.g. with very *large Fasciola*) it may be necessary to further bind the slides lightly with cotton.



Text-fig. 1. Method of flattening helminths for whole mount preparations. The specimen is dried superficially on filter-paper and placed on a slide (*S''*) with a dab of vaseline at either end. A second slide (*S'*) is pressed gently down on the specimen; the adhesive properties of the vaseline cause the slides to adhere firmly together.

Carmine staining

With some of the histochemical methods described below it is further necessary to stain nuclei with a red stain. Although a great many of the routine carmine stains are reasonably good, we believe that a relatively unknown variety—Gower's carmine (1939)—is worthy of more widespread use. It stains precisely, giving an unusual red colour with a slightly bluish tinge. It is prepared as follows:

Add 10 grs. carmine to 100 ml. of 45 % acetic acid, and bring it slowly to the boil; cool and filter. The residue on the filter-paper is acidified carmine. The staining solution consists of: acidified carmine, 1 g.; alum, 10 g.; distilled water 200 ml. Mix, dissolve by warming, and filter. Add a crystal of thymol.

In his original paper, Gower recommended differentiation by bleaching in Mayer's chlorine water (potassium chlorate and hydrochloric acid)—a somewhat violent method and one difficult to control. We prefer to differentiate more slowly in 0·5 % HCl in 70 % alcohol for 3–12 hr., depending on the size of the specimen. This variety is particularly recommended for platyhelminths in general.

HISTOCHEMICAL METHODS

(*a*) *Methods based on the presence of polyphenol oxidase*

The catechol technique: If cells containing polyphenol oxidase are incubated with catechol (DL-*o*-diphenol) as a substrate, *o*-quinone will be produced at the enzyme site, provided that the enzyme has been preserved during fixation, and this quinone will combine with adjacent protein molecules and 'tan' them—a light brown or reddish brown colour resulting. This method is the basis for a histochemical test for polyphenol oxidase (Smyth, 1954) which is particularly suitable for whole mount preparations. In the original technique only 70 % alcohol was used as a fixative for whole mounts.

We have now carried out further experiments to determine whether other fixatives can be used to preserve the enzyme. A number of *Fasciola* were fixed simultaneously in a range of fixatives for 12 hr., washed in water for 30 min. and incubated for 1 hr. in 0·1 % catechol in a water-bath at 40° C. They were then washed, upgraded, cleared and mounted. The results are shown in Table 1.

As was expected on theoretical grounds, the more corrosive of the fixatives destroyed the enzyme activity. Two of the results are surprising. First, tanning after formalin fixative is excellent, although perhaps not quite as precise as after 70 % alcohol. Secondly, after fixation in 5 % acetic acid no positive enzyme test was given by the vitellaria—indicating that the enzyme had been destroyed—but the hardening eggs in the uterus gave a strong reaction. The significance of these results is dealt with in the discussion.

Table 1. *Effect of fixation on the degree of tanning of the vitellaria of* Fasciola, *after incubation in* 0·1 % *catechol at* 40° C. *for* 90 *min.*

(The results reflect the degree of enzyme preservation after each fixative.)

Fixative	Degree of tanning	
	Uterus	Vitellaria
Bouin	None	None
Carnoy	Fair	Fair
10% formalin	Excellent	Excellent
5% acetic acid	Excellent	None
Absolute alcohol	Excellent	Excellent
90% alcohol	Excellent	Excellent
70% alcohol	Excellent	Excellent
$HgCl_2$ (sat. aq.)	None	None
Picric acid (sat.)	Weak	Weak
5% formol 1% acetic	Weak	Weak

The enzyme can withstand treatment with formalin, for specimens of *Fasciola* which had been kept in 10 % formalin for 12 days, on incubating with 0·1 % catechol—without washing—still gave a strong positive tanning reaction. This is an important result from the point of view of the helminthologist as it means that specimens may be stored in 10 % formalin for some days if necessary, although we have never attempted to determine the limiting period of storage.

In specimens successfully tanned by the catechol technique only the vitellaria and eggs are demonstrable, and these appear as brown to reddish brown, the colour depending on the nature of the protein being tanned; no other structure is stained in any way. Such preparations may be cleared and mounted and show up the arrangement of the vitellaria, together with its ducts, and the uterus in a remarkable manner (Pl. II, fig. 3). Alternatively, if desired, 'tanned' specimens may now be further stained by routine methods to show the remaining structures.

In the pseudophyllidean cestodes studied, the lability of the enzyme appears to vary considerably and in some forms is difficult to preserve. Thus, in *Diphyllobothrium latum*, the catechol technique reacts brilliantly on specimens fixed in 70 % alcohol—the vitellaria and uteri standing out from an almost colourless background (Pl. II, fig. 2). With formalin-fixed specimens, the reaction is considerably

weaker, a result in contrast to that obtained with *Fasciola*. In *Schistocephalus solidus* the catechol technique works reasonably well, but the crowding of the vitelline glands does not permit such a sharp result as with *Diphyllobothrium latum*.

In *Schistocephalus*, the distribution of the enzyme varies with the maturity of the proglottid, and this may be very strikingly demonstrated by means of the catechol technique. Pl. II, fig. 4, shows a strobila matured *in vitro* for 36 hr. in which the proglottids are just beginning to produce eggs; i.e. the vitellaria are rich in polyphenol oxidase and the uteri, which contain only a few eggs, appear almost colourless. Contrast this result with that of a strobila matured *in vitro* for 72 hr., in which the uteri are filled with eggs, as shown in Pl. II, fig. 6; here the vitellaria appear almost colourless, as most of the polyphenol oxidase has been carried into the uterus during egg formation; the uterus thus reacts brilliantly with catechol.

Catechol is unstable in alkaline solution, and it can be shown experimentally that for *Fasciola* the optimum enzyme activity is at pH 6·2. However, the enzyme has a wide pH range, and for routine purposes 0·1 % catechol in distilled water suffices, provided that it is freshly prepared on the day of use.

Reduced to its simplest, the technique recommended is as follows:

(1) Fix preferably in 70 % alcohol, but 10 % formalin may be used for trematodes. 1–3 days.

(2) Wash in water for 30 min.

(3) Place in 0·1 % catechol (freshly prepared) for 60–90 min. at 40° C., or 4–4½ hr. at room temperature (about 15° C.).

(4) Wash in water for 15 min.

(5) For vitellaria and uterus only—upgrade, clear and mount in balsam; vitellaria and uterus—reddish brown.

(6) For complete morphology—stain additionally in Gower's carmine (or other nuclear stain), 3–12 hr.

(7) Differentiate carmine in 0·5 % HCl in 70 % alcohol, 3–12 hr.

(8) Upgrade, clear and mount in balsam.

(*b*) *Methods based on the occurrence of phenolic substances*

The diazo technique. The majority of tests for phenols (argentaffin, chromaffin, sodium iodate, ferric chloride) are unsatisfactory at a morphological level, as they are either destructive or give colours which are microscopically unsuitable. In many cases, the diazo method, as used for argentaffin cell granules gives results equal to the catechol method. It is particularly useful in cases in which the enzyme may have been destroyed during fixation. The technique involves treating with a solution of a stable diazotate, of which a number are available. In our experience, the stable diazotate of 5-nitro-anisidine gives satisfactory results but must be freshly prepared before use. This salt is available under various names—Fast Red salt B (G. Gurr Ltd., 136 King's Road, London, S.W. 6); Fast Red salt V (Ciba, Basel); Kernechtrotsalz B (I. G. Farbenindustrie); Asorotsalz B (Bayer, Leverkusen); Naphthanil Diazo Red B (Du Pont de Nemours). Other stable diazotates may also prove satisfactory, but we have found the diazotates of *o*-amino-azotoluene

(Fast Garnet; Gurr) and of *o*-dianisidine (Fast Blue B salt; Gurr) not as effective as Fast Red salt B. A full list of salts available is given by Pearse (1953, p. 478).

The technique recommended is as follows:

(1) Fix in 70% or 90% alcohol; 1–3 days (or longer).

(2) Bring to water.

(3) Transfer to 1% Fast Red salt B, freshly prepared and filtered just before use; 10–40 min.

(4) Wash in water for 15 min.

(5) For shell-producing genitalia—dehydrate, clear and mount. Vitellaria, vitelline ducts and lower uterus—orange to red.

(6) For complete morphology—stain additionally in Gower's carmine for 3–12 hr.; destain 3–12 hr. in acid alcohol (0·5% HCl in 70% alcohol) upgrade, clear and mount.

The diazo technique works particularly well after alcohol fixation, but the colour obtained shows variation within the range—orange to bright red—depending on the nature of the phenolic materials in the vitelline cells. On theoretical grounds it may be expected that the recently formed egg-shell will lose its phenolic properties due to the formation of quinone and subsequent protein bonding, as it moves up the uterus. Thus the vitellaria and the freshly formed eggs react brilliantly, but the reaction decreases in intensity as the eggs pass up the uterus.

Alcohol fixation (70–90%) is in general recommended for the diazo technique, but some specimens fixed in formalin for a week have given excellent results, e.g. the monogenetic trematode *Diclidophora* (Pl. II, fig. 7); whereas others, e.g. *Fasciola*, have given poor results; the significance of these observations is not yet fully understood.

TECHNIQUES FOR SECTIONS

Methods that stain the egg-shell material may sometimes be useful for tracing out vitelline glands or ducts in sections, and so be of assistance in making reconstructions of female genitalia.

(*a*) *Catechol and diazo methods*

Specimens are fixed as in the previous section, but the times of treatment with catechol or Fast Red are approximately doubled. The treated worms are embedded in wax and sectioned, when the vitellaria, ducts and uterus will appear brown to red in section depending on the length of staining. It will be found that ducts which appear dark red in whole mounts at low-power level may appear only yellow or amber in sections at high power or oil-immersion level. Consequently, as indicated above, the treatment with catechol or Fast Red should be made more intense.

(*b*) *Bromo-phenol-blue method*

Mazia, Brewer & Alfert (1953) have shown that bromo-phenol-blue, in a mercuric chloride solution, links readily to proteins; whereas aqueous bromo-phenol-blue, without mercury, will bond with basic proteins only. On theoretical grounds, therefore, the vitellaria and the egg-shell should show a strong affinity for this dye in aqueous solution.

In practice this is often an elegant method, capable of giving the most precise and delicate staining of vitelline globules and egg-shells; but the intensity of staining is likely to vary with species, as it depends on the nature of the protein present.

Procedure: fix in Bouin, Carnoy or 10 % formalin; embed and section.

(1) Bring sections to water.

(2) Stain in 0·1 % aqueous bromo-phenol-blue; 10 min.

(3) Wash in 0·5 % acetic acid; 20 min.

(4) 'Blue' in tap water or buffer at pH 6; 3 min.

(5) Upgrade, differentiating in absolute alcohol if necessary.

(6) Mount in balsam.

Result: vitelline globules and eggs in lower uterus—blue.

This dye is quite unsuitable for whole mounts due to absorption on the cuticle which tends to obscure the genitalia.

(c) *Malachite green method*

It has been shown that the vitelline globules in many trematodes and pseudophyllidean cestodes have a marked affinity for aqueous malachite green (Smyth, 1951*a*, *b*). This affinity is shared with other quinone-tanning systems—such as byssus formation in *Mytilus* (Smyth, 1954). The material to which the dye molecules become attached was unknown, although on theoretical grounds it was likely to be one of the participants in quinone tanning—protein, phenol or polyphenol oxidase. It has now been shown that it is to basic proteins that the malachite green becomes bonded.

The method adopted to establish this point was as follows. A section of the cestode *Schistocephalus solidus* was stained by Mazia's method for proteins as indicated in the previous section. Several marked vitelline cells were drawn under oil immersion to show the distribution of the bromo-phenol-blue staining globules. The coverslip was removed and the bromo-phenol-blue removed by overnight extraction in absolute alcohol. The slide was then restained in malachite green, and remounted. The *same cells* were then redrawn, and it was found that the distribution of the malachite green staining globules was identical with those stained in bromo-phenol-blue. It may therefore be concluded that malachite green is similarly bonded to basic proteins.

The procedure for malachite green, slightly modified from the 1951 method is as follows:

(1) Fix in 10 % or 5 % formalin; 24 hr. Wash in running water 6–12 hr. Embed and section.

(2) Bring sections to water and counterstain nuclei in a red nuclear stain, e.g. 0·5 % neutral red—10 min. or Gower's carmine—2–12 hr. or 1 % safranin—5 min.

(3) Rinse in water.

(4) Stain in 0·5 % malachite green; 5 min.

(5) Upgrade 70 %, 90 % to absolute alcohol. Differentiate in absolute alcohol, the green will stream rapidly out of all tissues except the vitellaria and eggs in uterus.

(6) Counterstain in 1 % orange G in absolute alcohol; 5 sec. (optional).

(7) Rinse in absolute alcohol, clear and mount.

Result: nuclei, red; cytoplasm, pink and yellow; egg-shell material, green.

In the original technique, fixation for only 2 hr. was used. This works satisfactorily for many trematodes, but recent results suggest that the protein materials are much more labile in cestodes and require prolonged fixation to assure their retention in sections treated with aqueous reagents; 24 hr. fixation is therefore recommended for all helminths.

This technique is recommended for routine studies on helminth anatomy—particularly trematodes; as it usually facilitates the easy differentiation of the various ducts and glands as seen in sections. It is stressed that the degree of affinity for malachite green is likely to vary with species, depending on the nature of the protein present.

DISCUSSION

This paper is an attempt to show that several histochemical methods may be adapted for the demonstration of certain organ systems in whole mount preparations. It is particularly emphasized that these techniques are limited in their use to helminths producing a quinone-tanned egg, and cannot be adopted for use with groups such as cyclophyllidean cestodes where no true egg capsule of this type is formed.

Of the helminths investigated, the majority of trematodes and many pseudophyllidean cestodes may produce eggs of this type but there is accumulating evidence to suggest that there is a wide difference between different species. Thus the common trematodes—*Fasciola*, *Haplometra* and *Dolichosaccus*—give brilliant reactions with the catechol technique, whereas in *Gorgoderina* sp. (bladder of the frog) the vitellaria and eggs give consistently negative results. Again, of the pseudophyllidean cestodes examined, *Diphyllobothrium latum* gives outstanding results (Pl. II, fig. 2) with catechol, *Schistocephalus solidus* gives a reasonably good reaction, but another member of the family Diphyllobothriidae—*Diphyllobothrium* sp. (probably *dendriticum*)—has so far given unsatisfactory results with this reagent.

It is not possible then, at this very early stage in our knowledge of this field, to draw more than the most tentative conclusions as to how the various helminth groups will react to these methods. Helminths which have extensive vitellaria are likely, on theoretical grounds, to be producing a quinone-tanned egg, and it can be speculated that in addition to the monogenetic and digenetic trematodes and pseudophyllidean cestodes, many of the Cestodaria, and the tetraphyllidean and trypanorhynchan cestodes may also give satisfactory results with some of these techniques.

A negative result with catechol indicates the absence of polyphenol oxidase, so that in forms giving negative results, the egg must presumably be formed and hardened by some other mechanism as yet unknown. In insects, where the cuticle is commonly a tanned protein, recent work suggests that an alternative method may also occur. Thus, Pryor (1955) has produced evidence to indicate that in the puparium of *Calliphora*, the cuticle may be tanned by aminophenols derived from

tryptophane instead of dihydroxyphenols derived from tyrosine. It is possible that this method may also occur in helminth eggs, but no evidence of this is yet available.

Workers unfamiliar with histochemical techniques will find *Fasciola* or *Diphyllobothrium latum* excellent material for preliminary testing of these methods. The effects of various fixatives or different incubation times may readily be compared by cutting a single specimen into several pieces and clearing and mounting each fragment after treatment. For both the catechol and diazo techniques, 70% alcohol is the most satisfactory general fixative, although the catechol method works well in trematodes after formalin fixation.

This latter result is surprising, for all available evidence suggests that the $-NH_2$ groups in the protein molecules are concerned in the linking with the quinone nuclei, but since $-NH_2$ groups are usually considered to be blocked by formol (Pryor, 1940; Pearse, 1953), the mechanism may not be as simple as was previously considered. After fixation in 5% acetic acid, the catechol reaction is no longer given by the vitelline glands, suggesting the destruction of the enzyme, but a strong reaction is still given by the eggs in the uterus. This might be interpreted as indicating, as Hackmann (1953) suggests, that the *protein of the enzyme itself becomes tanned*—and that when such a tanning process has taken place the groups concerned in the phenol oxidizing mechanism may be protected in some way so that the active part of the enzyme is unaffected by acetic acid.

As a routine method for demonstrating the morphology of trematodes we have found the catechol method followed by Gower's carmine generally superior to the diazo method. Catechol has the advantage of being cheap and readily procurable, and the colour produced—being due to protein tanning—is virtually indestructible in any of the normal laboratory reagents.

This paper is primarily to stress the advantages of a histochemical *approach* to the gross morphology of helminths, and it has merely dealt with two methods which have been found to give satisfactory results with some forms. More precise methods for phenols, proteins or the polyphenol oxidase or other enzyme systems may be developed later which may be more readily applied to helminths. The extension of histochemical methods to cover other organ systems—ovaries, testes, excretory canals and gut—could transform the present rather empirical methods for whole mount preparations, into a series of rational techniques which would greatly assist the working out of the detailed anatomy of helminth groups.

SUMMARY

1. The egg-shell in many trematodes and pseudophyllidean cestodes is a quinone-tanned protein; and histochemical methods for polyphenol oxidase or for phenols may be used to demonstrate regions producing or containing shell material (i.e. the vitellaria, its ducts and the uterus) in whole mount preparations.

2. Specimens so treated may be further stained by routine whole mount methods. Gower's carmine is particularly recommended.

3. 70% alcohol was found to be the best general fixative for these methods.

4. These histochemical methods—with certain modifications—may also be used for demonstrating the egg-shell producing regions in sections.

5. Because of their affinity for basic proteins, bromo-phenol-blue and malachite green are suitable for staining these regions in sections. These stains are unsuitable for whole mounts.

6. There is some evidence to suggest that all trematodes and pseudophyllidean cestodes do not form and harden their egg-shell by quinone tanning; consequently, some forms may give negative or unsatisfactory results with these methods.

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EXPLANATION OF PLATE II

Fig. 1. *Fasciola hepatica*. Fixed 70% alcohol, treated by the catechol technique followed by Gower's carmine.

Fig. 2. *Diphyllobothrium latum*. Fixed 70% alcohol and treated by the catechol technique. The vitellaria and uterus, which are rich in polyphenol oxidase, show up reddish brown.

Fig. 3. *Fasciola hepatica*. Treated by the catechol technique as in Fig. 1, but the later treatment with carmine has been omitted. Note the distribution of polyphenol oxidase is limited to the vitellaria and uterus.

Fig. 4. *Schistocephalus solidus*. Proglottids just becoming mature, treated by the catechol technique, showing concentration of polyphenol oxidase in the vitellaria. Contrast with Fig. 6.

Fig. 5. *S. solidus*. Treated by the diazo technique. Fixed in 70% alcohol; treated with Fast Red salt B.

Fig. 6. *S. solidus*. Treated as in fig. 4. Mature specimen with large numbers of eggs in uterus. Note that, compared with fig. 4, the enzyme has now shifted from the vitellaria into the uterus to take part in the formation of the egg-shell.

Fig. 7. *Diclidophora merlangi*. Treated by the diazo technique, followed by Gower's carmine. Fixed 10% formalin 10 days; treated with Fast Red salt B.

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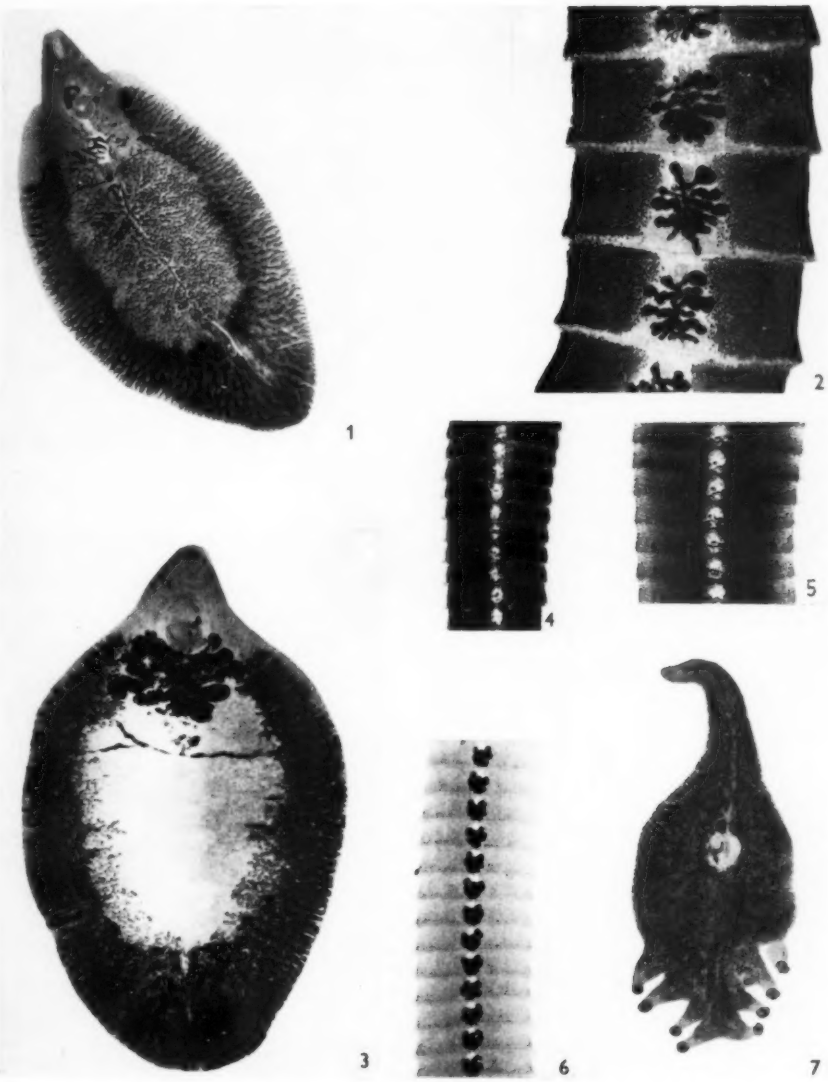

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MODIFICATION AND ELIMINATION OF THE BACTERIAL FLORA IN CULTURES OF *ENTAMOEBA INVADENS* RODHAIN, 1934

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I. INTRODUCTION

The first report of the successful cultivation of a species of *Entamoeba* was that by Cutler (1918), who described a method by which he claimed to have maintained *E. histolytica* in culture for several months. Cutler's results, however, could not be confirmed by other workers, and the validity of his claim has been questioned (Dobell, 1919). Dobell attributed the first successful cultivation of a species of *Entamoeba* to Barret & Smith (1923, 1924), who devised a method by which both *E. barreti* and *E. ranarum* could be maintained *in vitro*. Soon afterwards, a medium was devised for *E. histolytica* by Boeck & Drbohlav (1925), whose method has since been used, with modifications, by many workers. Owing to the clinical interest in *E. histolytica*, the growth *in vitro* of this species has been investigated more thoroughly than that of any other *Entamoeba*, but the methods used for *E. histolytica* are generally applicable to nearly all other species of *Entamoeba*. Although many modifications and simplifications of the culture media originally used are now available, little advance has been made in one respect; as in the original cultures, the amoebae grow only in the presence of living bacteria, and repeated attempts to maintain sterile cultures of *E. histolytica* for prolonged periods have, with few exceptions, been unsuccessful. Within the last few years, Shaffer and his colleagues (Shaffer & Sienkiewicz, 1952; Shaffer, Sienkiewicz & Washington, 1953) have reported that *E. histolytica* can be cultivated without living organisms, in medium containing minced chick embryo, and this method has been used successfully by Rees and his colleagues (Rees, Baernstein, Reardon & Phillips, 1953).

The presence of a mixed bacterial population, derived from the intestinal flora, in cultures of *Entamoeba*, is an undesirable variable factor in any experimental work on the growth of the amoebae. In the absence of axenic cultures (Dougherty, 1953), cultures of amoebae growing with a simplified, known flora should be used, preferably monoxenic cultures; and monobacterial strains of *E. histolytica*, or

strains growing with only *Trypanosoma cruzi*, are now widely used. In addition, the preparation of monoxenic strains is an important step towards obtaining axenic cultures. Various methods have been devised for producing monobacterial strains, and, essentially, comprise the elimination of the bacterial flora, and the inoculation of sterile amoebae or cysts into medium containing only one species of bacterium.

At one time, it was hoped that monoxenic strains might provide material suitable for studying certain aspects of the physiology of the amoebae, but only limited success has been achieved with such strains of *E. histolytica*; in particular, the work of Rees *et al.* has shown that great caution is needed in interpreting the results of metabolic studies using monobacterial strains, as synergism may occur between the amoebae and the bacteria (Rees *et al.* 1953). Precise information concerning the growth requirements and metabolism of *Entamoeba* will be obtained only with axenic cultures of the amoebae.

As previously mentioned, most of the investigations on the cultural requirements of *Entamoeba* have been made using cultures of *E. histolytica*. It is possible, however, that this species does not provide the ideal material, particularly for preliminary work on the growth requirements of the amoebae in axenic culture. Lamy (1948*a*, *b*) and Miller (1951, 1953), working independently, isolated axenic strains of *E. invadens* in essentially the same medium, containing liver or chick embryo; the later report from Miller states that his axenic strain has been maintained for over 2 years in saline containing untreated, sterile liver. An investigation of the behaviour of *E. invadens in vitro* was begun by the writer several years ago, in order to compare the cultural characteristics of this species with those of the morphologically identical *E. histolytica*, and also because it seemed possible that *E. invadens* might be a suitable organism for studies on the growth requirements of a species of *Entamoeba* in axenic culture.

Strains of *E. invadens* growing with a known bacterial flora were therefore required, and an account is given here of the methods by which strains growing with simplified flora were obtained; monobacterial strains were later prepared, and ultimately, a strain of amoebae growing without bacteria or other living cells. At the time when this work was contemplated, a report by Rao (1951) had just appeared, in which he claimed to have sterilized cysts of *E. histolytica*, obtained from faeces or cultures, by treatment with chemicals. The strain of *E. invadens* available produced large numbers of cysts *in vitro*, and it was therefore decided to attempt to sterilize cysts according to the method described by Rao, and to use these sterile cysts to initiate monobacterial strains of amoebae.

II. MATERIAL AND METHODS

The strain of *E. invadens*, from which all the experimental strains originated, was isolated by Professor J. Rodhain of Antwerp, and has been maintained at the Molteno Institute since 1947. The amoebae in this strain were growing in HSre+S (see below), with a mixed bacterial flora, and produced cysts readily; no attempt was made to identify the species of bacteria present, but they included a slime-

producing organism, which rendered the manipulation of the cultures difficult, as the deposit of amoebae and starch at the bottom of the cultures tended to form a glutinous mass.

The media used to maintain the strains of *E. invadens* growing with bacteria were the diphasic horse serum—Ringer-eggwhite (HSre of Dobell & Laidlaw, 1926); liquid horse serum—Ringer (hs), consisting of one part of sterile inactivated serum mixed with seven parts of sterile Ringer's solution; and an infusion of egg-yolk in saline-phosphate (eysaph), prepared according to the method of Balamuth & Sandza (1944). Strain ST was maintained in tubes of eysaph, to which a slice of chick or rat liver was added before the medium was sterilized by autoclaving. Sterile powdered rice starch (S) was added to all the media before inoculation, and cultures were maintained at 24° C., by subculture at intervals of approximately 3 weeks.

The axenic strain of *E. invadens* was maintained in the medium used by Miller (1953) for maintaining axenic cultures of the organism. 0·75 % saline was tubed in 10 ml. amounts, and sterilized by autoclaving; to each tube was then added a slice of liver, removed, with sterile precautions, from a chick or rat. The tubes of saline-liver were then incubated at 37° C. for 24 hr.; any grossly contaminated tube was readily detected in this way. Very occasionally, the liver was contaminated by slow-growing bacteria, which were detected only after the tubes had been inoculated with amoebae and incubated for several days or weeks at 24° C.; such cultures were discarded. It was found that chick liver was contaminated more often than liver from rats, and rat liver was subsequently used as the standard ingredient. The liver was generally obtained from normal rats, but occasionally rats which had recovered from an infection with *Trypanosoma lewisi* had to be used; no difference was detected in the growth-promoting properties of liver from normal and infected rats. When the axenic strain was first isolated, anaerobic conditions were produced in each tube of saline-liver by placing a plug of absorbent cotton-wool, soaked in 0·4 ml. of 20 % pyrogallol and 0·7 ml. of 20 % sodium carbonate, above the original cotton-wool plug; the tube was then closed with a rubber bung. Later, it was found more satisfactory to seal the cultures, after inoculation with amoebae, with vaseline, sterilized previously by autoclaving.

The reagents used for eliminating bacteria from cysts or amoebae were all sterilized before use: Ringer's solution (Dobell & Laidlaw, 1926), 0·85 % sodium chloride, 0·002 % mercuric chloride, 0·02 % potassium permanganate, 0·02 % acriflavine, 0·2 % gentian violet and 0·25 % neutral red, by autoclaving; and N/5 sodium bicarbonate by Seitz filtration. N/10 hydrochloric acid was tubed in sterile tubes, and boiled and cooled immediately before use.

Cysts were washed by suspension in Ringer's solution or saline, followed by centrifugation. Treatment with chemicals was carried out by suspending cysts in approximately 5 ml. of solution, and centrifuging down at the end of treatment. All these operations were carried out, with aseptic precautions, at room temperature. After treatment with chemicals, cysts were inoculated into medium seeded with a known species of bacterium—i.e. into medium containing a 24 hr. culture of the required bacterium.

All the strains of bacteria, except *Bacterium coli* FA′ (see McConnachie, 1955), were selected from species maintained at the Molteno Institute on nutrient agar slopes.

Sterility tests were carried out in tubes of 2 % glucose broth and Brewer's thioglycollate medium; on slopes or plates of nutrient agar, blood agar or Levine's medium; and in Robertson's meat broth, prepared from minced horse heart and dispensed in screw-cap containers. Anaerobic tests were made in a container filled with nitrogen.

III. DIBACTERIAL STRAINS OF *ENTAMOEBA INVADENS*

The bacterial flora of the original strain of *E. invadens* was first simplified by treatment with acid. Cysts from several cultures were pooled and washed; they were then treated with N/10-HCl, which was neutralized, after varying intervals of time up to 30 min., with N/5-$NaHCO_3$, neutral red being used as an indicator of pH. After being washed, the cysts were inoculated into HSre + S seeded with *Bact. coli* 15. Amoebae were obtained from all batches of cysts treated in this way, and although acid treatment did not sterilize the cysts, it did eliminate the slime-producing organism, and possibly some others, as the bacterial growth was visibly less in cultures obtained from acid-treated cysts. Such cultures were more easily handled than those of the untreated strain, as the deposit of amoebae and starch no longer formed a glutinous mass.

The bacterial flora was further simplified by treatment with chemicals, according to the method described by Rao (1951). Cysts from several cultures were pooled and washed two or three times. They were then treated with 0·002 % $HgCl_2$ for 45 min., washed twice and treated with 0·02 % $KMnO_4$ for 15 min. After further washings, the cysts were treated overnight (approximately 20 hr.) with 0·02 % acriflavine. Finally, the cysts were thoroughly washed until no traces of acriflavine were visible in the washing solution, and suspended in approximately 1 ml. of Ringer's solution; portions of this suspension were inoculated into culture medium seeded with the required species of bacterium, and into bacteriological media to test for sterility.

Cultures inoculated with treated cysts were examined for periods up to 3 weeks after inoculation, and amoebae were first observed from 3 to 15 days after inoculation. Cultures of amoebae were obtained in several types of media, and in the presence of different single species of bacteria. The histories of the strains of *E. invadens* produced by the above method are given in Table 1. All these dibacterial strains were voluntarily discarded after being cultivated for several years, during which observations were made on their behaviour *in vitro*; in particular, factors affecting the growth and encystation of strains A and M were investigated (McConnachie, 1955).

This chemical treatment of cysts resulted in a high mortality rate, and the cytoplasm of many treated cysts was brown and granular; sometimes cysts so treated failed to yield cultures, and in order to obtain a higher percentage of positive cultures, a less drastic method of treatment was sought. The gross morphological

changes observed in some of the cysts were largely the result of treatment with $KMnO_4$. When cysts of strains A and C were treated with $HgCl_2$ for 45 min., followed by treatment with acriflavine for approximately 20 hr., no bacterial growth was produced in glucose broth, or on nutrient or blood agar inoculated with the treated cysts and incubated aerobically or anaerobically at 24 or 37° C. Although treatment with $HgCl_2$ and acriflavine appeared to kill a fairly high proportion of the cysts, cysts so treated produced cultures of amoebae more regularly than when $KMnO_4$ was also used, and the former treatment was later used as the standard method of eliminating *Bact. coli* from the cysts of strains A, C, D and GV.

Table 1. *The history of dibacterial strains of* Entamoeba invadens *derived from cysts treated with chemicals*

Strain	Strain from which cysts were obtained	Chemicals used to treat cysts	Medium in which cysts were hatched	Bacteria in hatching medium
A	Original strain, after treatment with N/10-HCl	$HgCl_2$, $KMnO_4$ + acriflavine	HSre + S	*Bact. coli* 15
C	Original strain	$HgCl_2$, $KMnO_4$ + acriflavine	HSre + S	*Bact. coli* 15
D	A	$HgCl_2$, $KMnO_4$ + acriflavine	eysaph + S	*Bact coli*. FA′
F	A	$HgCl_2$, $KMnO_4$ + acriflavine	hs + S	*Bact. coli* FA′
M	A	$HgCl_2$ + acriflavine	hs + S	*Bacillus megatherium*

Organism *x* has been identified in the flora of strains A, C, D and M, and was presumed to be present in strain F.

Treated cysts used to initiate strains A, C, D, F and M were inoculated into glucose broth and plated on nutrient agar; no growth was observed in these media after aerobic and anaerobic incubation for 3–6 days at 24 and 37° C. Confirmation of the sterility of the treated cysts was sought by examination of the bacterial flora of the strains derived from these cysts. Direct microscopical examination of cultures, of various ages, of strains A, F and D revealed the presence of only coliform organisms; Gram-stained films of the flora of these strains showed only Gram-negative rods, identical with those in stock cultures of *Bact. coli* 15 and *Bact. coli* FA′. Media in which strains A, F and D were growing were plated on blood agar and Levine's medium, and incubated aerobically and anaerobically at 24 and 37° C.; the colonies produced were identical with those on plates inoculated with stock cultures of *Bact. coli*, and in numerous examinations, no contaminating species was ever detected.

When cultures of strain M were examined, however, a small rod-shaped organism was found, in addition to the large rods of *Bacillus megatherium*. Gram-stained films of such cultures showed large Gram-positive rods and rods which had lost their Gram-positive staining reaction, both typical of *B. megatherium*; in addition, numerous small rods, which gave a weak Gram-negative reaction, and which bore a marked resemblance to *Bact. coli*, were found. Strain M had been derived from

strain A, and the treated cysts used to initiate it had produced no growth when inoculated into glucose broth; these cysts had therefore been sterilized of the *Bact. coli* known to be present in the flora of strain A. Routine bacteriological tests on culture medium had never revealed the presence of bacteria, and it was unlikely that the coliform organism present in strain M originated from contaminated medium; it seemed more probable that this bacterium had been introduced with the chemically treated cysts used to initiate the strain. As these cysts had been obtained from strain A, presumably the organism was also present in strain A, but its marked resemblance to *Bact. coli* would have made it difficult to distinguish the two species in Gram-stained films of the bacterial flora of strain A.

More stringent bacteriological tests made it possible to isolate this coliform organism. Cysts from cultures of strain A were treated with $HgCl_2$ and acriflavine, and inoculated into glucose broth and blood agar, incubated aerobically and anaerobically at 24° C.; the treated cysts were also inoculated into Robertson's meat broth, a naturally anaerobic medium, and incubated at 24° C. No bacterial growth was observed in the glucose broth after 33 days' incubation, or in blood agar after 4 days. In the Robertson's meat broth, however, slight fermentation was observed after 7 days, and Gram-stained films revealed a small rod-shaped organism, of doubtful staining reaction; some rods appeared to be Gram-positive, while the majority were Gram-negative. This bacterium, designated organism *x*, is identical with that originally observed in the cultures of strain M.

Organism *x* has been isolated, in Robertson's meat broth, on many occasions from chemically treated cysts from strains A, C and D, and its presence is easily recognized in cultures of strain M, although it has not been possible to isolate it in pure culture from this strain, as the spore-bearing *B. megatherium* proved resistant to treatment with chemicals. No attempt was made to isolate organism *x* from strain F, but as this strain was derived from strain A, organism *x* was presumably present in the former strain also.

Attempts to identify organism *x* were unsuccessful.* Growth occurred at 24–37° C. in only Robertson's meat broth and Brewer's thioglycollate medium, or in semi-solid agar, McCartney's meat broth and nutrient broth, all containing 5% serum; thus organism *x* may be a microaerophilic bacterium, rather than a strict anaerobe. Cultures can be maintained indefinitely in Robertson's meat broth, but die out after a few subcultures in Brewer's medium. An attempt was made to determine the biochemical reactions of the organism by inoculating from cultures in semi-solid serum agar into standard bacteriological media containing 5% serum; the tests were run for 5 weeks at 30° C., but, in all, the results were negative.

Morphologically, the organism consists of straight or slightly curved rods, 0·7 × 2–4 μ in the early stages of growth, later becoming pleomorphic and forming long filaments; no spores or capsules have been found. In young cultures, the rods are Gram-positive, but rapidly become Gram-negative; no beading is shown with Neisser's stain, and the rods are non-acid fast.

* I am indebted to Mr G. C. Ingram, of the Low Temperature Research Station, Cambridge, for carrying out many of these tests.

Its resistance to treatment with chemicals suggested that organism *x* might form spores. Tests were therefore made to determine if organism *x* formed heat-resistant spores in the media used for maintaining cultures of *E. invadens*. Both young and old mixed cultures of *Bact. coli* FA′ and organism *x* in eysaph + S were heated to 80° C. for 30 min. and to 65° C. for 15 min., and then inoculated into Robertson's meat broth; no bacterial growth was obtained. Mixed cultures of *B. megatherium* and organism *x* were also heated to 80° C. for 30 min.; such treatment destroyed organism *x*, but the spore-bearing *B. megatherium* survived. It was therefore concluded that organism *x* did not form spores in the cultures of *E. invadens*, and since spores have never been found in stained films of pure cultures of organism *x*, it is probably not a spore-forming bacterium.

IV. MONOBACTERIAL STRAINS OF *ENTAMOEBA INVADENS*

To produce a strain of *E. invadens* growing with a single species of bacterium, it was necessary to eliminate organism *x* from the flora of one of the dibacterial strains. The use of antibiotics was contemplated, but as organism *x* did not grow in simple bacteriological media, its sensitivity to antibiotics could not readily be determined. Dobell (1952) and Neal (1952) eliminated Gram-positive spore-bearing anaerobes from strains of *E. histolytica* by using gentian violet, and it seemed possible that organism *x* might also be susceptible to treatment with gentian violet.

A substrain (designated strain GV) of strain D was maintained in the presence of gentian violet according to the method devised by Dobell, and confirmed by Neal. HSre medium was prepared with only 1 ml. of Ringer-eggwhite in each tube; a tube of this medium was inoculated with 5 drops of a 24 hr. broth culture of *Bact. coli* FA′, and incubated at 37° C. for approximately 24 hr. Immediately before inoculating amoebae into the culture, starch and 0·5 ml. of 0·2 % gentian violet were added to the medium, the dye being carefully mixed with the Ringer-eggwhite. Amoebae from a culture of strain D in HSre + S were then inoculated into the medium, and the culture was incubated for approximately 24 hr. at 24° C. Amoebae from this culture were subcultured into fresh HSre + S + gentian violet, containing a 24 hr. growth of bacteria, as before, and this procedure was repeated daily for a total of seventy-two subcultures in the presence of gentian violet. During the first subcultures of strain GV, few, or occasionally no, amoebae were found on microscopic examination, but some survived, and after thirty-three subcultures in medium containing 0·5 ml. of 0·2 % gentian violet, the growth of the amoebae improved slightly, and a higher concentration of dye (1·0 ml. of 0·2 % gentian violet/1 ml. of Ringer-eggwhite) was used. After eighteen successive cultures in this concentration, the growth of the amoebae became very poor, and the dye was reduced to its original strength, but the last thirteen subcultures were also made in the higher concentration of gentian violet. Thus the amoebae were maintained for seventy-two subcultures in a concentration of at least 0·5 ml. of 0·2 % gentian violet in 1·0 ml. of medium, while for certain periods twice this concentration of dye was used.

Although the amoebae in the cultures containing gentian violet were few in number and small, they ingested starch and moved actively; the cytoplasm of some, which were otherwise normal in appearance, was stained a light purple, and stained bacteria were frequently ingested. Cysts were never found while strain GV was maintained in the presence of the dye. Many stained bacteria were found in the cultures, particularly in those containing the higher concentration of dye, but daily inoculation of the amoebae into fresh medium containing a 24 hr. growth of *Bact. coli* FA′ ensured that they were maintained in the presence of living bacteria for at least part of every 24 hr. period.

It was hoped that the multiplication of the individual bacteria of organism *x*, which were introduced into strain GV in the original inoculum from strain D, would be inhibited by the gentian violet, and that these bacteria would eventually be killed during their maintenance in the presence of the dye, so that the flora of strain GV would comprise only *Bact. coli* FA′. Proof that organism *x* had been eliminated could not be obtained by a simple procedure, and the flora of strain GV was not examined until the strain had been maintained in gentian violet for a considerably longer period than Dobell or Neal found sufficient to eliminate anaerobes; these workers obtained strains of *E. histolytica* free from anaerobes after maintaining them in the presence of gentian violet for 20 and 33 days. When strain GV had been maintained in HSre + S + gentian violet for 72 days, it was inoculated into HSre + S medium, in which it was subsequently maintained while tests were made to determine the composition of its bacterial flora.

A method was required by which it could be determined whether organism *x* was still present in the flora of strain GV; *Bact. coli* FA′ was known to be present, and, as previously mentioned, organism *x* could not be detected in Gram-stained films of mixed cultures, nor, since it did not grow on solid media, by plating techniques. Organism *x* had been isolated many times from chemically treated cysts from strains A and D, and it was considered that if chemical treatment of cysts from strain GV failed to reveal the presence of organism *x*, whereas identical treatment of cysts from strain A permitted of its isolation, then it could be concluded that organism *x* was not present in strain GV.

Two samples of cysts, from cultures of strains A and GV in HSre + S, were each treated with $HgCl_2$ and acriflavine, according to the method previously described. After each batch of cysts had been thoroughly washed, it was suspended in approximately 1 ml. of Ringer's solution, and inoculated into Robertson's meat broth and aerobic glucose broth; the cyst suspensions were also plated on blood agar, incubated aerobically. The inoculated media were then incubated at 24° C. for 3 weeks. Bacterial growth was observed after 7 days in Robertson's medium inoculated with the treated cysts from strain A; Gram-stained films showed a small rod-shaped organism, which proved to have the characteristics of organism *x*. No bacterial growth was produced, in 21 days, in any of the media inoculated with treated cysts from strain GV. The experiment was repeated three times, each with the same result; organism *x* was always isolated from the treated cysts from strain A, but never from those obtained from strain GV. The growth of organism *x* in Robertson's meat broth inoculated with strain A cysts was not always detected

within 7 days, but was invariably detectable within 14 days. In addition, organism *x* has been isolated on eight out of nine occasions when cysts from strains A or D were treated with $HgCl_2$ and acriflavine, but it has never been detected in seven attempts to isolate it from similarly treated cysts from strain GV. On the only occasion when organism *x* was not isolated from treated cysts from strain A, the bacterium may have been eliminated by chance, or the Robertson's medium may have differed slightly from that normally used, and been unsuitable for the growth of organism *x*.

It is considered that the results obtained in these experiments prove that organism *x* was not present in the flora of strain GV; as this strain was derived from strain D, whose flora comprised *Bact. coli* FA′ and organism *x*, organism *x* must have been eliminated during the maintenance of strain GV in gentian violet, and the amoebae of strain GV were now growing in the presence of *Bact. coli* FA′ alone. Strain GV was maintained *in vitro* for nearly 2 years, and then discarded; its growth and encystation were very similar to those of the dibacterial strains of *E. invadens* growing with *Bact. coli* (15 or FA′) and organism *x*.

A second monobacterial strain of *E. invadens* (strain ST), growing with *Staphylococcus albus*, was prepared from cysts produced in cultures of strain GV. Preliminary experiments had indicated that the growth of *E. invadens* with *Staph. albus* alone was very variable in normal eysaph + S or HSre + S, and it was difficult to maintain such a monobacterial strain in these media; therefore, eysaph medium enriched with autoclaved chick liver was used for the maintenance of strain ST. Cysts from strain GV were sterilized, as previously described, by treatment with $HgCl_2$ and acriflavine, and inoculated into eysaph + autoclaved liver + S, seeded with *Staph. albus*; the treated cysts hatched within 11 days. The sterility of the treated cysts was established by inoculating them into aerobic glucose broth and Robertson's meat broth, and by plating on blood agar; no bacterial growth was produced after incubation at 24° C. for 3 weeks. The purity of the flora of strain ST was confirmed by regular examination of Gram-stained films of cultures; these films showed only Gram-positive cocci. The growth of strain ST was less heavy than that of strain GV, and cysts were found only occasionally, but the growth of the strain did not diminish during the 20 months for which it was maintained.

V. AN AXENIC STRAIN OF *ENTAMOEBA INVADENS*

A strain of *E. invadens* (strain N), growing without bacteria or any living cells, was prepared by treating strain ST with antibiotics, to which the amoebae were not sensitive. The amoebae from two cultures of strain ST in eysaph + autoclaved liver + S were inoculated into a tube of saline containing a slice of sterile chick liver, and approximately 1000 units of penicillin (crystalline sodium penicillin G) and 1000 units of streptomycin (dihydrostreptomycin sulphate) per 1 ml. of medium; the culture was incubated at 24° C., with an alkaline pyrogallol plug to absorb the oxygen. The strain was maintained for seven subcultures in medium containing the antibiotics, by subinoculation at intervals of approximately 3–4 days. During this time, few amoebae were present, but they continued to multiply and moved actively. The 8th subculture was made into medium without antibiotics,

and subinoculations from the 9th subculture, made into aerobic Saboraud's maltose medium (to test for possible contamination with fungi) and glucose broth, and into Robertson's meat broth, were all negative after 14 days' incubation at 24° C. A vaseline seal was substituted for the alkaline pyrogallol plug at the 10th subculture, and rat liver replaced chick at the 13th subculture. The liver in the medium tends to disintegrate slightly after several weeks, but its growth-promoting properties are apparently unimpaired; saline-liver is frequently used after being stored for several weeks at 8° C.

Strain N has now been maintained for over 14 months in saline-liver medium, by subculture at intervals of approximately 3 weeks; antibiotics are never added to the medium, and microscopic examination of cultures and sterility tests, made regularly into glucose broth and Robertson's meat broth, have never revealed the presence of bacteria. The growth of the amoebae in cultures of strain N is markedly less than in cultures of *E. invadens* growing with bacteria, but growth has been very consistent, and may have improved slightly since the strain was first isolated. Most cultures survive for at least 6 weeks, and one culture still contained living amoebae after 10 weeks' incubation at 24° C.; no attempt has been made to determine the maximum time for which cultures of strain N may survive. Strain N is also maintained in saline-liver containing starch, but although the amoebae readily ingest starch grains, their growth appears no heavier than in cultures without starch. Amoebae from cultures, both with and without starch, contain glycogen, as shown by staining with iodine.

The amoebae of strain N are slightly larger than those in cultures growing with *Bact. coli*, or with a mixed bacterial flora; this increase in size is probably correlated with their slower growth rate (McConnachie, 1954*b*). For some unknown reason, the cytoplasm of the amoebae in the axenic cultures is often vacuolated, some amoebae containing numerous large vacuoles; these vacuolated amoebae are otherwise quite normal, however, as they move actively, and have been observed dividing.

Cyst formation in strain N is comparatively rare, compared with that in most of the di- and monobacterial strains of *E. invadens*. Microscopic examination of many cultures has failed to reveal the presence of cysts, but occasionally, isolated cysts have been found in cultures showing a comparatively good growth of amoebae. As cysts are usually found amongst a mass of disintegrating liver tissue, they are not easily detected, and it is difficult to investigate their structure; chromatoids appear to be present, but it has not yet been established whether viable, four-nucleate cysts are produced.

VI. CONCLUSIONS

The method devised by Rao (1951) for sterilizing cysts of *E. histolytica* failed to sterilize those of *E. invadens*, obtained from a strain growing with a mixed bacterial flora; this confirms the findings of Dobell (1952) and Neal (1952), that treatment with chemicals does not consistently sterilize the cysts of intestinal amoebae. Spore-bearing anaerobes proved resistant to treatment with acriflavine (Dobell), and to the treatment devised by Rao (Neal), and in the present experiments, Rao's method failed to kill not only the spore-bearing *B. megatherium*, but also organism *x*, which does not appear to form spores.

As chemical treatment killed all the bacteria, except organism *x*, which were present in the flora of the original strain of *E. invadens*, it is probable that spore-bearers were not present in this flora, or that under the conditions of culture, spores were not formed. Neal found that spore-bearing bacteria were present in the flora of all the strains of *E. histolytica* which he examined, such bacteria being normal inhabitants of the human intestine. The bacterial flora of the original strain of *E. invadens* was presumably derived from that which accompanied the organism at the time of its isolation from a reptile by Rodhain, but the detailed history of the strain prior to the present work is not known. It is possible that any spore-bearers originally present died out during the maintenance of the strain of amoebae *in vitro*, or that spore-bearing bacteria are not always found in the reptilian intestine.

The presence of organism *x* hampered early attempts to produce monobacterial strains of *E. invadens*. It was considered, however, that the dibacterial strains would be suitable for experimental work, as each was growing with organism *x* and one other known species of bacterium; any differences observed in their behaviour could therefore be attributed to the influence of the different species of bacteria in each strain. In particular, studies were made on the growth and encystation of strains A and M (McConnachie, 1954*b*, 1955). Strain F was used for tests on the action of drugs on *E. invadens in vitro*; preferably, such tests should be made on a monobacterial strain, and the results obtained with strain F were therefore confirmed later, using the monobacterial strain GV (McConnachie, 1954*a*).

Strain GV of *E. invadens*, which was growing with *Bact. coli* FA′ alone, formed cysts in HSre + S, and thus differed from Dobell's monobacterial strain of *E. histolytica*, growing with *Bact. coli* A′, which never formed cysts (Dobell, 1952). Cysts formed in strain GV were readily sterilized by treatment with chemicals, and thus provided a source from which other monobacterial strains could be prepared. The monobacterial strain ST, on the other hand, provided a means of readily obtaining sterile amoebae, as the flora of this strain was very sensitive to antibiotics.

The axenic strain of *E. invadens* has been maintained, ever since its isolation, in the medium devised by Miller to support the growth of *E. invadens* without bacteria, and this medium appears adequate for the continued maintenance of such axenic strains. It is, however, lacking in certain factors, as the growth of the axenic strain is poor compared with that in mixed cultures of amoebae and bacteria.

The constitution of the medium in which strain N is growing does little to indicate the nutritional requirements of *E. invadens*. But, as pointed out by Nakamura (1953), it does not really matter if a complex medium has to be used for first maintaining pure cultures of *Entamoeba*; once cultures free from other micro-organisms have been obtained, it should be possible progressively to simplify the medium, and so, in time, to determine some of the growth requirements of the amoebae. One of the chief values of these axenic cultures of *E. invadens* is that they provide a ready source of sterile amoebae with which to test the growth-promoting properties of other media; in the past, the lack of a constant source of sterile amoebae has been one of the difficulties in attempts to devise suitable media for maintaining axenic cultures of *Entamoeba*.

VII. SUMMARY

1. Cysts, formed in a strain of *E. invadens* growing with a mixed bacterial flora, were sterilized of all but one species of bacterium (organism *x*), by treatment with chemicals. Strains of *E. invadens* derived from these cysts were prepared, growing with organism *x* and either *Bact. coli* or *B. megatherium*.

2. A monobacterial strain of *E. invadens*, growing with *Bact. coli* alone, was obtained by maintaining a dibacterial strain, growing with organism *x* and *Bact. coli*, for 72 days in medium containing gentian violet.

3. A monobacterial strain of *E. invadens*, growing with *Staph. albus*, was prepared from cysts, produced in the monobacterial strain of *E. invadens* + *Bact. coli*, and sterilized by treatment with chemicals.

4. An axenic strain of *E. invadens* was prepared by maintaining the monobacterial strain of *E. invadens* + *Staph. albus*, in saline-liver medium containing antibiotics. This axenic strain has now been maintained for over 14 months in saline containing untreated, sterile liver, but no antibiotics.

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MORPHOLOGICAL AND TAXONOMIC STUDIES ON MAMMALIAN TRYPANOSOMES

VIII. REVISION OF *TRYPANOSOMA EVANSI*

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With a STATISTICAL ANALYSIS by J. W. TREVAN, F.R.S.

(With 10 Figures in the Text)

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I. INTRODUCTION

Among the pathogenic mammalian trypanosomes, those causing the disease commonly known as Surra (and by numerous other local names) are of particular interest to protozoologists and veterinary workers. Because of the multiplicity of hostal strains and their wide geographical range, the host-parasite relations, affinities and taxonomic status of these trypanosomes have for a long time been a matter of controversy, which can only be settled by a thorough revision.

In the Old World, Surra has an extremely wide geographical distribution in countries with hot or warm-temperate climates, especially where arid deserts and semi-arid steppes are prevalent. It extends in longitude from about 15° W. to 125° E., but its distribution in latitude is more difficult to define. In Africa the disease extends from the Atlantic and Mediterranean coasts as far as 15° N. in French West Africa, but in the East it spreads farther south, to about 13° N. in the Anglo-Egyptian Sudan and almost to the Equator in Somaliland. On the Eurasian continent its distribution is uneven and patchy. In the Near and Middle East Surra has been reported from Israel, Lebanon and Syria, the northern half of the Arabian Peninsula, Asia Minor, Iraq and Iran. In Europe, apart from an isolated record from Bulgaria, the Trans-Volga region of the Soviet Union is the area chiefly affected by this disease, which reaches the northernmost point of its

distribution near Kuibyshev (= Samara, *ca.* 53° N.), whence it passes into Soviet Middle Asia, and extends into Iran, India, Burma, the Malay Peninsula, Indo-China and parts of southern China. It also occurs in islands of the Indian Ocean (Mauritius, Réunion), which represent the southernmost points of its distribution (*ca.* 20° S.), and in Malaysia (Indonesian islands, Philippines). Finally, this disease has also found its way into the New World, where it is prevalent in Central and South America. The area of distribution of Surra thus includes the Palaearctic, Ethiopian, Madagascar, Oriental and Neotropical zoogeographical regions. Here it should be noted that, since the records upon which the above data are based belong to different periods and could not be checked, some of them might not apply to present conditions.

Surra affects a variety of domestic and wild mammals, especially camels, horses, donkeys, cattle, pigs and dogs, as well as Asiatic buffaloes, elephants, tapirs, and (in Mauritius) deer. The chief symptoms of the disease are fever and anaemia, which may be followed by oedema and cachexia, while its course may be acute or chronic, frequently terminating in death, or it may be mild or even symptomless: in the latter case the infected animal is a healthy carrier. The severity of the disease varies according to the degree of susceptibility of the host, but in various parts of the world a given host-species may be affected in different ways. Thus, in Indo-China and Java the disease is acute in horses but mild in cattle, whereas in the Anglo-Egyptian Sudan and Somaliland horses are refractory to infection, cattle are only slightly affected, but camels are highly susceptible. On the other hand, in North Africa horses and camels are equally susceptible. The Surra group of trypanosomes therefore comprises strains differing in their effect upon various hosts present in the same locality (Hoare, 1943).

In strains from all known geographical and hostal sources, the trypanosomes appear to be morphologically similar to the causative organism of the classical Indian Surra, originally named *Trypanosoma evansi*, being typically represented by thin trypanosomes, corresponding to the slender and intermediate forms in members of the *Brucei* group. Furthermore, Surra is everywhere transmitted by Tabanid flies mechanically, i.e. without undergoing any development in the vector. In spite of the common features uniting the parasites and the diseases caused by them in different countries, some observers were so impressed by variations in the clinical course of the infections and by the host-restriction exhibited by diverse strains, that they sought to differentiate the causative organisms accordingly. Some years ago much importance was attached—especially by the French school initiated by Laveran and Mesnil—to cross-immunity as a method for distinguishing Surra strains, with the result that a number of strains were separated from *T. evansi* as independent species and varieties. Although the recognition of the existence of immunologically distinct intraspecific strains in blood protozoa has discredited the diagnostic value of the cross-immunity test, some of the names created on this basis are still in use. Other strains have been separated from *T. evansi* on grounds of morphological, hostal or clinical differences. The classification of various strains in distinct taxa has resulted in a multiplicity of names for the Surra trypanosomes, of which over fifteen are extant (see list on p. 161),

producing the impression of a significant difference between the strains in question. Although the independent status of these strains and the validity of the names attached to them have been repeatedly challenged, no attempt has hitherto been made to solve this question by a thorough revision of these trypanosomes.

The idea of a revision of the Surra trypanosomes developed gradually. Originally the object of my investigation—started some 20 years ago—was limited to the question of the affinities of *T. evansi*. Though it is generally admitted that it is allied to members of the *Brucei* group, the closeness of this relationship was not evident until I discovered in some strains of this trypanosome a marked polymorphism, comparable to that in *T. brucei*, and briefly reported this observation (Hoare, 1940*a*, 1952). In the course of this work, I managed to collect abundant material from various sources, which enabled me to pursue the study of polymorphism in many strains. However, as the work progressed, other interesting problems emerged, such as the unexpected finding of mutant akinetoplastic strains, the behaviour of this trypanosome in tsetse-flies, and its evolutionary history: these were also dealt with and duly described (Hoare & Bennett, 1937, 1939; Hoare, 1940*a*, *b*, 1948*a*, 1954*a*). These studies diverted me for some years from the main line of investigation, but it was not neglected, for during that period I continued observations on the polymorphism of *T. evansi*. Finally, when this problem was satisfactorily solved, I decided to use the available material for a morphological and statistical study of the Surra trypanosomes. In this paper, therefore, I propose to make a revision of the morphology and taxonomy of these trypanosomes, with special reference to their affinities. Although the material at my disposal was by no means fully comprehensive, it represented the greatest number of strains from the widest variety of sources that had ever been brought together and subjected to a comparative biometrical study. It is hoped that this revision of *T. evansi* will not only be of academic zoological interest, but will also provide data of practical value in veterinary medicine.

II. MATERIALS AND METHODS

The material for the present investigation consisted of thin blood films, stained by Giemsa's or Leishman's modifications of Romanovsky's method. The greater part of this material was kindly supplied, at my request, by workers in many countries throughout the area of distribution of Surra. The films were obtained from diverse mammalian hosts: in most of the cases from naturally infected animals, but in some cases—when these were unobtainable—from early passages in experimentally infected laboratory animals. The rest of the material was derived from strains maintained by me in laboratory animals and kept under continuous observation for many years. The sources of all the material used are given in Table 1, together with the other data, viz. scientific names borne by the strains, labels attached to them by me, dates when the films were made, and references to papers containing further information about the strains in question.

Here it is necessary to define some of the terms used in this paper. In medical and veterinary parasitology, the word *strain* denotes a closely related group of individuals that perpetuate their characters in successive generations. It is repre-

Table 1. *Material used in p*

Strains	Names		Hosts	Coun
A	*T. soudanense, T. evansi* var. *mborii*	Cameline	Camel	A.E. Sudan
B	*T. soudanense, T. evansi* var. *mborii*		Camel	A.E. Sudan
C	*T. soudanense, T. evansi* var. *mborii*		Camel	A.E. Sudan
Ca	*T. soudanense, T. evansi* var. *mborii*		Gerbil *ex* cattle from camel	A.E. Sudan
Cb	*T. soudanense, T. evansi* var. *mborii*		Gerbil *ex* donkey from camel	A.E. Sudan
Cc	*T. soudanense, T. evansi* var. *mborii*		Mice *ex* camel	A.E. Sudan
D	*T. evansi, T. evansi* var. *mborii*		Camel	British Som
E	*T. ninae kohl-yakimov, T. evansi* var. *su-auru*		Camel	Uralsk, U.S.
F	*T. ninae kohl-yakimov, T. evansi* var. *su-auru*		Camel	Turt-Kul, U
G	*T. ninae kohl-yakimov, T. evansi* var. *su-auru*		Camel	Ashkhabad,
*T**	*T. soudanense, T. evansi* var. *mborii*		Camel	A.E. Sudan
*U**	*T. soudanense, T. evansi* var. *mborii*		Camel	A.E. Sudan
*AA**	*T. soudanense, T. evansi* var. *mborii*		Camel	A.E. Sudan
V	*T. ninae kohl-yakimov, T. evansi* var. *su-auru*		Camel	Middle Asia
H	*T. annamense*	Equine	Horse	Lai-Chau, In
J	*T. evansi*		Horse	Philippines
K	*T. evansi*		Horse	Mukteswar,
L	*T. evansi*		Guinea-pig *ex* horse	Java
M	*T. marocanum*		Guinea-pig *ex* horse	Morocco
Na	*T. evansi*		Mouse *ex* horse	Bulgaria
Nb	*T. evansi*		Mouse *ex* horse	Bulgaria
O	*T. evansi*		Mule	Mauritius
*W**	*T. marocanum*		Horse	Morocco
*X**	*T. marocanum*		Horse	Morocco
P	*T. annamense*	Bovine	Cattle	Nhatrong, I
Q	*T. evansi*		Cattle	Mauritius
R	*T. annamense*		Buffalo	Hanoi, Indo
*Z**	*T. annamense*		Cattle	Nhatrong, I
S	*T. evansi*	Canine	Dog	Baghdad, I
*Y**	*T. marocanum*		Dog	Morocco

* Strains marked with an asterisk have not been treated statistically.

† Dates when blood films were made.

‡ I am indebted to the gentlemen named for kindly providing the trypano laboratory animals.

§ These substrains have been maintained by me in London.

Table 1. *Material used in present work*

losts	Countries	Dates†	Sources‡
	A.E. Sudan	vi. 1934	Dr S. C. J. Ben
	A.E. Sudan	i. 1934	Dr S. C. J. Ben
	A.E. Sudan	iv. 1937	Dr S. C. J. Ben
ttle from camel	A.E. Sudan	xii. 1937	Dr S. C. J. Ben
nkey from camel	A.E. Sudan	vi. 1937	Dr S. C. J. Ben
el	A.E. Sudan	xi–xii. 1937	C.A.H.§
	British Somaliland	x. 1935	Govt. Vet. Offic
	Uralsk, U.S.S.R.	v. 1935	Dr D. N. Sassu
	Turt-Kul, U.S.S.R.	vi. 1935	Prof. N. I. Cho
	Ashkhabad, U.S.S.R.	1936	Mr L. Bogorodi
	A.E. Sudan	iii. 1934	Dr S. C. J. Ben
	A.E. Sudan	vi. 1934	Dr S. C. J. Ben
	A.E. Sudan	1938–1942	C.A.H.§
	Middle Asia, U.S.S.R.	1936	Prof. N. I. Cho
	Lai-Chau, Indo-China	1938	Dr H. Galliard
	Philippines	1913	Dr M. B. Mitzm
	Mukteswar, India	viii. 1934	Mr W. Taylor
x horse	Java	v. 1934	Prof. C. Bubber
x horse	Morocco	*ca.* 1926	C.A.H.§
rse	Bulgaria	v. 1939	Dr P. Pavlov
rse	Bulgaria	vii. 1939	C.A.H.§
	Mauritius	i. 1934	Dr A. R. D. Ad
	Morocco	1917	Dr H. Velu
	Morocco	1917	Dr H. Velu
	Nhatrong, Indo-China	1939	Dr Jacotot
	Mauritius	1934	Dr A. R. D. Ad
	Hanoi, Indo-China	i. 1939	Dr C. Toumanc
	Nhatrong, Indo-China	1939	Dr Jacotot
	Baghdad, Iraq	iii. 1938	Mr C. R. Chadv
	Morocco	1917	Dr H. Velu

t been treated statistically.

for kindly providing the trypanosome material, either in the form of preparation

y me in London.

Sources‡	Remarks
r S. C. J. Bennett	—
r S. C. J. Bennett	Ref. Hoare & Bennett (1937) = case I (akinetoplastic)
r S. C. J. Bennett	Ref. Hoare & Bennett (1939) = case V (akinetoplastic)
r S. C. J. Bennett	Substrain passaged from *C*
r S. C. J. Bennett	Substrain passaged from *C*
A.H.§	Substrain (polymorphic sample) passaged from *C*
ovt. Vet. Officer	—
r D. N. Sassuchin	—
rof. N. I. Chodukin	—
r L. Bogoroditsky	—
r S. C. J. Bennett	—
r S. C. J. Bennett	—
A.H.§	Ref. Hoare (1954) = strain *NS*
rof. N. I. Chodukin	—
r H. Galliard	—
r M. B. Mitzmain	—
r W. Taylor	—
rof. C. Bubberman	—
A.H.§	—
r P. Pavlov	Ref. Pavlov & Guenev (1939)
A.H.§	Substrain (polymorphic sample) passaged from *Na*
r A. R. D. Adams	Ref. Adams (1935)
r H. Velu	Ref. Barotte (1925)
r H. Velu	Ref. Barotte (1925)
r Jacotot	—
r A. R. D. Adams	Ref. Adams (1935)
r C. Toumanoff	—
r Jacotot	—
r C. R. Chadwick	—
r H. Velu	Ref. Barotte (1925)

of preparations or in infected

sented by a population of organisms from a given individual host, while populations passaged from such a population are known as *substrains*. A strain thus corresponds to the concept of a *deme* (from $\delta\eta\mu os$ = community, population), a term introduced by Gilmour & Gregor (1939) for a single population of related individuals within a specified taxon (such as species). It has also been proposed to distinguish the nature of a deme by an appropriate prefix, e.g. topodeme—occupying any specified geographical area, ecodeme—occupying any specified ecological habitat (including, in the case of parasites, their hosts). If this terminology is applied to the present investigation, it will be seen that the available material on thirty populations of *T. evansi* (*sensu lato*) comprised twenty-six strains or demes and four substrains, represented both by topodemes and ecodemes (cf. Table 1).

The statistical study of variation in samples from twenty-two strains (= demes) was based on measurements of the total length of the trypanosomes, made by drawing (with the aid of a camera lucida at $\times 2500$) a line running through the middle of the body of each trypanosome, from the posterior end to the tip of the flagellum, and measuring this line with a divider set at $\frac{1}{20}$ in. (= *ca.* $0{\cdot}5\mu \times 2500$).

III. POLYMORPHISM

As already mentioned in the Introduction, in typical strains the Surra trypanosomes are represented by thin forms, indistinguishable from the slender and intermediate forms in the *Brucei* group, whereas the stumpy forms characteristic of the polymorphic trypanosomes are usually absent. That the absence of stumpy forms in *T. evansi* is not absolute is evident from a number of published records of the occurrence of such forms in various strains of this species.

(*a*) *Criteria*

Since the forms found in this species resemble those in trypanosomes of the *Brucei* group, and because the writings of some authors reveal a misconception regarding the scope of polymorphism in this group, it is necessary, in order to avoid confusion, to give an exact definition of the criteria used to distinguish the different component forms. As far as I was able to ascertain, Dutton & Todd (1903) were the first to recognize in *T. gambiense* two distinct types, which they called the 'long' and the 'stumpy' forms, but the existence of trimorphism in this species was first noted by Minchin (1908), who stated that (*a*) 'the slender form is of great length and has a very long free flagellum', (*b*) 'the stumpy form is short', and (*c*) 'the ordinary form is...intermediate between the two extreme forms'. More precise morphological characters were given, in the case of *T. brucei*, by Bruce, Hamerton, Bateman & Mackie (1910), according to whom (*a*) 'the long and slender [forms] have a narrow extended posterior extremity...and a long free flagellum', (*b*) 'the short and stumpy have a rounded posterior extremity, somewhat like the head of a turtle', and (*c*) 'the intermediate have a short free flagellum, a posterior extremity resembling that of the short and stumpy form, but narrower'. Though not mentioned in the text by Robertson (1912), the same differential characters are

depicted by her for the two thin forms of *T. gambiense*. Almost identical morphological criteria for the thin forms of *T. rhodesiense* were recently established by Fairbairn & Culwick (1946), who had apparently overlooked the original description given by Bruce *et al.* (*loc. cit.*). According to these authors, the slender forms have a long narrow posterior end, with the kinetoplast some distance (up to 4μ) anteriorly to it, while the intermediate forms have a blunter posterior end, with—judging from their figures—the kinetoplast near this end. Another feature, which I purposely omitted above, is the presence or absence of a free flagellum in the stumpy forms, regarding which there is no unanimity. Thus, the presence of a short free flagellum is noted by Minchin (1908) and Robertson (1912), while Bruce seemed to be undecided, for in a series of publications (with or without associates) between 1910 and 1915 the stumpy forms are said to lack a free flagellum, but in one paper (Bruce, Harvey, Hamerton & Bruce, 1913*b*) its presence is definitely admitted and its length (0·2–4μ) is given. There is no doubt at present (as noted also by Fairbairn & Culwick, 1946) that in stumpy forms of trypanosomes of the *Brucei* group a short free flagellum may be present, though in most cases it is absent. Although the polymorphism of this group is now a well-established fact, until quite recently the degree of segregation of the three component forms was not clear. Thus, Bruce *et al.* (1910), Robertson (1912, 1913), and Lavier (1928) believed that they were not sharply separated but formed a continuous series connected by transitional forms. Nevertheless, the following ranges of length for the differentiation of these forms were given by Bruce *et al.* and Robertson in the above papers (shown under *a* and *b* respectively): stumpy (*a*) 13–21μ, (*b*) 14–20μ; intermediate (*a*) 22–24μ, (*b*) 20–24μ; slender (*a*) 25–35μ, (*b*) 23–33μ. These arbitrary mensural criteria were the only ones available until Fairbairn & Culwick (1946) demonstrated statistically that the length distribution of *T. rhodesiense* could be resolved into three distinct curves corresponding to the stumpy, intermediate and slender forms, thereby introducing for the first time exact criteria for their separation.* This classification was later confirmed and extended by Mr A. T. Culwick and Dr K. C. Willett, who made a statistical analysis of two populations of *T. brucei* and one *T. rhodesiense*, as well as of the length distributions previously recorded by Bruce and Robertson for five populations comprising all the three polymorphic species: in each of these cases they succeeded in fitting the total lengths into three curves corresponding to the three component forms. The results of this laborious work, which is unpublished, were kindly communicated to me in a letter (1947), together with the charts, one of which is reproduced here in Fig. 2.

It is thus evident that the species of the *Brucei* group (*T. brucei*, *T. rhodesiense*, *T. gambiense*) are characterized by a trimorphism, determined by the existence in these trypanosomes of three phases, which are separable morphologically and statistically into stumpy, intermediate and slender forms. The heterogeneity of these trypanosomes therefore conforms to the genetical concept of polymorphism,

* Although the earlier statistical study of trypanosomes of the *Brucei* group by Maynard (1915) produced similar results, this author attributed the multi-modal curves to mixed infections.

which is defined as the co-existence within a species of a number of distinct forms or phases, the difference between which is revealed by a multi-modal frequency curve of variability. Since there is an overlap in their dimensions (see Fig. 2), the epithets 'short' and 'long', previously attached to the stumpy and slender forms respectively, are misleading and should be discarded, as already suggested by Lavier (1928). From the definition given above it also follows that other morphological variants, such as the aflagellar thin forms noted by some authors (e.g. Joyeux & Sautet, 1941) in *T. evansi* and erroneously used by them as criteria of polymorphism, need not be taken into consideration.

(*b*) *Previous records*

Having clarified the concept of polymorphism in the *Brucei* group, we now turn to a consideration of this phenomenon in *T. evansi*, in which the problem is reduced to the presence or absence of stumpy forms. These forms were first noted—and one was depicted—by Bruce (1911), but they were so rare that he felt justified in regarding *T. evansi* as monomorphic. A 'very rare "short" [=stumpy] form' is also illustrated by Fry (1911), and their occasional occurrence in this species was mentioned by Balfour (1914)—in both cases in cameline strains from the Anglo-Egyptian Sudan. A more precise study of polymorphism, in strains of *T. evansi* from Mauritius and Morocco, was carried out by Lavier (1933), who detected one stumpy form among 2000–3000 (=0·03–0·05 %) trypanosomes examined, and provided figures of these forms. In the cases cited above the proportion of stumpy forms was so insignificant as to be negligible, thus justifiying the view held in the past that *T. evansi* was practically a monomorphic species. It is true that Velu (1918 and in Barotte, 1925) found a high degree of dimorphism in strains from Morocco, but his main differential criteria were the presence or absence of a free flagellum. When these strains were re-examined by me (in the original films kindly lent by Dr H. Velu), the proportion of stumpy forms in them proved to be comparable to Lavier's figures (see strains *W*, *X*, *Y* in Tables 1 and 2). It is curious that the only record of an appreciable degree of polymorphism in *T. evansi*, by Fiori, Delanoë & Delanoë (1915, 1916), has unaccountably escaped notice in the literature. Although these authors referred to the Moroccan strains studied by them as dimorphic merely on the grounds that they contained trypanosomes with and without a free flagellum, among the figures illustrating the latter there are also about a dozen typical stumpy forms.

(*c*) *Present investigation*

From the foregoing review of previous work it is evident that in *T. evansi* a variable, but mostly insignificant, proportion of stumpy forms does occur from time to time. Since in many cases the strains examined had been maintained in laboratory animals, it was conceivable that—like trypanosomes of the *Brucei* group under similar conditions—they might have lost some of the polymorphism originally present in their natural hosts, as already suggested by Bruce (1914), Lavier (1933) and Fairbairn & Culwick (1946). Bearing in mind this possibility, I decided to re-investigate this question in material from natural hosts of *T. evansi*, and succeeded in obtaining a representative collection of blood films of nineteen individual

animals, from ten localities extending over the range of distribution of Surra, from Morocco to the Philippines. The hosts comprised camels (7), horses (5), mule (1), cattle (2), buffalo (1), and dogs (2); in addition to these, one equine strain (*L*) was represented by a first passage infection in a guinea-pig (for sources, etc., see Table 1). In these preparations I carefully examined from 1000 to 5000 trypanosomes, with the results shown in Table 2, from which it is seen that no stumpy forms could be detected in eleven animals, while in the other eight they were present in from 0·05 to 0·40 % of the trypanosomes examined, the average percentage for the total of 31,000 trypanosomes being 0·05, i.e. of the same order as in previous records, based chiefly on infections in laboratory animals. (These results have been briefly mentioned in a previous paper: Hoare, 1940*a*).

Table 2. T. evansi: *polymorphism in samples examined once**

Strains†	No. examined	Percentage stumpy forms	Strains†	No. examined	Percentage stumpy forms
B	1000	0	*H*	1000	0·10
U	2000	0·05	*J*	5000	0·10
T	1000	0	*L*	2000	0
D	1000	0	*O*	3000	0
E	1000	0	*P*	1000	0
G	1000	0	*Z*	1000	0·40
V	1000	0	*R*	1000	0·30
W	1000	0·10	*Y*	2000	0
X	1000	0·10	*S*	1000	0
K	4000	0·05			
			Total	31,000	0·05

* Strains (*Cc*, *Nb*) kept under continuous observation are dealt with separately.
† For description of strains see Table 1.

Since the data on polymorphism described above were based on the characteristics of the trypanosomes at the particular time when the blood film was taken, i.e. on the examination of a single random sample, it was interesting to ascertain whether the extreme rareness of stumpy forms in *T. evansi* was a constant feature, persisting throughout the infection. This could only be verified in strains kept under continuous observation, and the opportunity to do so presented itself later in the course of this investigation, when I secured three strains (*Nb*, *AA* and *Cc*), which were maintained and studied by me in the course of many years, with the results described below.

Strain *Nb* was isolated in 1939 from an infected horse in Bulgaria by Dr P. Pavlov (described by Pavlov & Guenev, 1939), who kindly sent me a substrain in infected mice, which has been maintained in these animals up to the present. In the course of 4 years (1939–43) blood films of this strain were regularly examined in each passage-animal: in some of the earlier passages for 3–12 days in succession, in others at intervals of 4–5 days, but subsequently only once a month. In each slide at least 100 trypanosomes were inspected for the presence of stumpy forms, the total number thus examined in about 500 preparations exceeding 50,000 trypanosomes. Among these, typical stumpy forms (Fig. 3, *s*) were detected on four separate occasions (in 1939), when 2, 6, 25 and 6 % respectively were present.

Strain *AA* (formerly *NS*), which was described in previous papers dealing with the loss of the kinetoplast in *T. evansi* (Hoare, 1954*a*; Hoare & Bennett, 1939), was isolated by Dr S. C. J. Bennett in 1938 from an infected camel in Kordofan, Anglo-Egyptian Sudan, and sent in a gerbil to London, where it has been maintained by me in mice up to the present. Blood films of this strain were regularly examined for about 5 years (January 1938–September 1942): in some passages daily for 3–25 days, in most cases at intervals of 4–5 days (when new animals were subinoculated), but from the end of 1942 onwards only once a month. Each time a minimum of 100 trypanosomes were scrutinized for the presence of stumpy forms, the total number of trypanosomes observed in about 600 preparations being over 60,000. Among these, typical stumpy forms (Fig. 3, *p*) were seen on eight occasions (between 1938 and 1940), reaching a maximum of only 2 %.

It is thus seen that even after prolonged and repeated observations over periods of several years, in the course of which upwards of 100,000 trypanosomes were examined, the presence of stumpy forms in *Nb* and *AA* was revealed only in four and eight samples respectively, but the proportion of stumpy forms (2–25 %) seen on these occasions was considerably higher than in any of the strains examined previously (see p. 136). In view of the inconstant and sporadic occurrence of polymorphism demonstrated in strains *Nb* and *AA*, the total absence or negligible numbers of stumpy forms, reported for strains examined only once, finds a ready explanation.

Strikingly different results were obtained with *Cc*, which is a substrain of *C*, representing a mutant akinetoplastic population isolated by Dr Bennett in 1937 from a naturally infected camel in Gedaref, Anglo-Egyptian Sudan, and therefore closely related—if not identical—to strains *A*, *AA* and *B* from the same locality and host (cf. Table 1). The akinetoplastic condition of *C* was fully dealt with by Hoare & Bennett (1939: case V) and Hoare (1954*a*: strain *SAK*). Strain *C* was first studied in the original camel and in subsequent passages through camel, horse, oxen, rats and gerbils. Gerbils of the 5th passage were sent to London, where the substrain *Cc* has since been maintained in passages through mice. Blood films from these animals were examined regularly in the course of 17 years (April 1937–April 1954): usually at intervals of about 1 week (when subinoculations were made), but in some passages for 5–19 days in succession. On each occasion at least 100 trypanosomes were examined for the presence of stumpy forms, the total number of trypanosomes seen in 1036 preparations (= 1018 passages) exceeding 104,000.

Unlike the strains of *T. evansi* examined by me previously, *Cc* exhibited a remarkable and persistent degree of polymorphism, as briefly reported in a preliminary note (Hoare, 1952). In the course of these observations, typical stumpy forms (Fig. 3, *o*, *q*, *r*, *t*) were seen on about 250 occasions, in proportions varying from isolated individuals to 61 %. The polymorphism of this substrain was at its height during the first nine years, when the maximum numbers of stumpy forms (20–61 %) were recorded, whereas during the last 8 years their numbers declined to from 2 to 6 %, only on two occasions rising to 12–13 %. This fluctuation was noted not only in films studied at weekly intervals but also in those examined daily. Thus, in the 25th passage (1937), examined for 19 days, the percentage of stumpy

forms varied from day to day as follows: 0, 0, 42, 0, 0, 6, 6, 13, 31, 12, 4, 6, 4, 9, 14, 3, 5, 9, 0. One of the most interesting peculiarities of this strain—hitherto unknown in *T. evansi*—was the common occurrence among the stumpy trypanosomes of so-called 'posterior-nuclear' forms in which the nucleus, instead of occupying a more or less central position in the body, is displaced to the posterior end (Fig. 3, *v*, *w*, *x*). In place of this cumbersome double epithet, I propose to adopt for these forms the term 'posteronuclear', introduced by Lavier (1927). Posteronuclear forms were frequently observed during the first 8 years (1937–45), after which they were seen only occasionally. The proportion of posteronuclear forms fluctuated considerably at different times, with a minimum of 3·2 % and a maximum of 66·6 % of the total number of stumpy forms present. However, more than 6 % posteronuclear forms were found only in eighty-eight films, whereas in 153 the percentage was lower, reaching only 1 % in seventy preparations. Here it may be noted that the wide fluctuation in the relative numbers of stumpy (including posteronuclear) forms, observed in my strains at different times, is another feature which *T. evansi* has in common with trypanosomes of the *Brucei* group, in which this fluctuation has been described by many authors (e.g. Robertson, 1912, 1913; Stephens & Fantham, 1912, 1913; Reichenow, 1921; Lester, 1933; Fairbairn & Culwick, 1946).

In view of the high proportion of stumpy forms revealed by prolonged observations on strains *Nb*, *AA* and especially *Cc*, as contrasted with the negligible numbers observed in random samples by previous observers and myself, the polymorphism of *T. evansi* has now been demonstrated beyond all doubt. In order to forestall a possible criticism that the polymorphic strains attributed here to *T. evansi* might actually belong to a member of the *Brucei* group, it must be emphasized that they were isolated from typical cases of Surra, in localities outside the area of distribution of tsetse-flies. Moreover, it was demonstrated experimentally that none of these strains was capable of developing in *Glossina* (Hoare, 1940*a*: strains *A*, *B*, *C* were identical with *Cc*, *AA*, *Nb* respectively). Likewise, there are no grounds for suspecting an accidental substitution of *T. evansi* by trypanosomes of the *Brucei* group maintained in the same laboratory, for every precaution was taken to prevent such an accident, and, in particular, because *Cc*, being totally akinetoplastic, was a 'marked' strain, and therefore could not have been confused with any other species.

Since the polymorphism in strains *Cc* and *Nb*—including the occurrence of posteronuclear forms in the former—proved to be of the same order as that in members of the *Brucei* group, it was decided to ascertain, by the method devised by Fairbairn & Culwick (1946) and by Culwick & Willett (1947, *in litt.*) for trypanosomes of this group, whether the length distributions of *T. evansi* could likewise be resolved into distinct curves corresponding to the three morphological components present. For this purpose I selected in both of these strains polymorphic samples and measured in each the lengths of 400 trypanosomes, at the same time classifying and marking each individual, according to the morphological form to which it belonged. Though most of the trypanosomes could be assigned to one or another of the three recognized types (stumpy, intermediate or slender), some individuals showed mixed

characters, either of stumpy + intermediate forms (Fig. 3, *m*, *n*) or of intermediate + slender ones (Fig. 3, *g*, *h*): such trypanosomes were designated *transitional* forms.

The sample of *Nb* belonged to the 16th mouse passage (July 1939), when the different forms were present in the following percentages: stumpy 35·25, intermediate 28·50, slender 27·50, transitional 8·75. For strain *Cc*, two samples were pooled, one belonging to the 22nd (November 1937), the other to the 25th mouse passage (December 1937). In the combined samples the different forms were

Table 3. T. evansi: *statistical constants for measurements of length of component forms in two polymorphic samples*

Strains	Forms	Mean lengths (μ)	σ	No. measured
Cc	Stumpy	19·6	1·4	106
	Intermediate	20·7	1·8	109
	Slender	24·9	2·7	158
	Total*	22·0	3·2	400
Nb	Stumpy	16·8	1·5	141
	Intermediate	19·5	1·6	114
	Slender	23·0	1·9	110
	Total*	19·4	3·0	400

* The totals include transitional forms which do not fit in the other types.

present in the following percentages: stumpy 26·50, intermediate 27·25, slender 39·50, transitional 6·75. The statistical constants for these samples are given in Table 3. When the frequency distributions of lengths of the three forms (stumpy, intermediate and slender) in the samples of *Cc* and *Nb* were plotted, they produced in both cases three distinct overlapping curves, shown in Fig. 1. These results show a close resemblance to the curves illustrating polymorphism in species of the *Brucei* group, described by Fairbairn & Culwick (1946) and by Culwick & Willett (*in litt.*), as seen from Fig. 2 (*T. brucei*), reproduced from one of the charts made by the last-named observers. From Fig. 1 it is noticeable that, although the curves for *Cc* and *Nb* provide statistical evidence of trimorphism in *T. evansi*, some of them are bi- or multi-modal, indicating a certain degree of heterogeneity, which might be due either to an admixture of individuals not conforming to the type in question (like the transitional forms noted above), or to some unknown physiological factors affecting the measurements of the trypanosomes. Dr Trevan has subjected this diversity to probit analysis, the results of which are discussed by him in the Appendix (p. 165), to which the reader is referred for details.

(*d*) *Morphology*

The morphological features of *T. evansi* have been familiar since its discovery by Steel in 1885. As mentioned before, this species is typically represented almost exclusively by thin trypanosomes, comprising slender and intermediate forms, which are indistinguishable from the corresponding forms in species of the *Brucei*

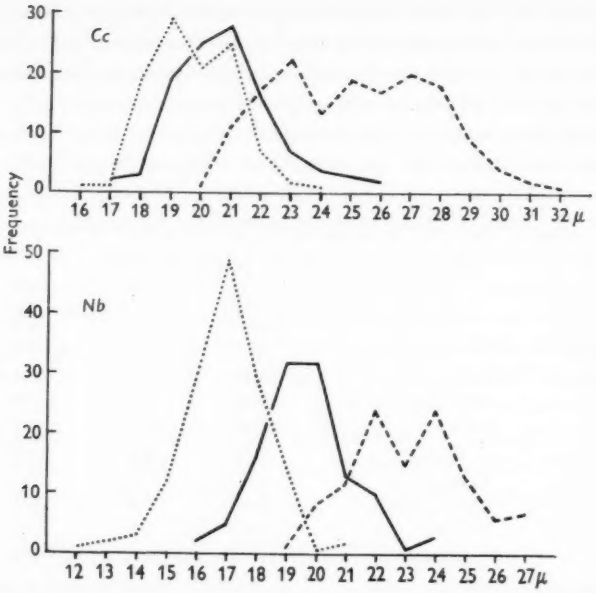

Fig. 1. *T. evansi.* Resolution of polymorphic samples in substrains *Cc* (top: 373 trypanosomes) and *Nb* (bottom: 365 trypanosomes) into three morphological types: stumpy (.....), intermediate (———) and slender (– – – – –).

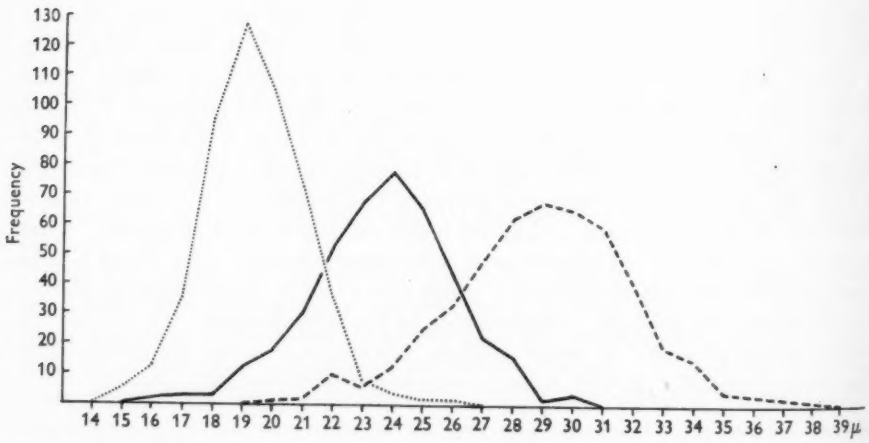

Fig. 2. *T. brucei.* Resolution of strain 'Shinyanga I' (1386 trypanosomes) into three morphological types: stumpy (.....), intermediate (———) and slender (– – – –). (Adapted from chart by Culwick & Willett, 1947: *in litt.*)

group. The slender forms have a long free flagellum and a more or less drawn out narrow posterior extremity, which may be rounded or truncated, with the kinetoplast situated at some distance from the tip (Fig. 3, *a–f*). The intermediate forms have a shorter free flagellum and a short, frequently pointed, posterior extremity, with the kinetoplast lying near this end (Fig. 3, *i–l*). In addition to these, there are always encountered some individuals showing mixed characters, on account of which they cannot be assigned to either of the thin trypanosomes, and are therefore referred to as transitional forms (Fig. 3, *g*, *h*). In the strains which came under observation in this study, the scanty occurrence of true stumpy forms, occasionally noted in random samples of *T. evansi*, has been fully confirmed. When present, stumpy forms usually make their appearance sporadically, and are therefore more likely to be detected in strains examined repeatedly, as in the case of *Cc*, *AA* and *Nb*. Judging from the results obtained with the last two strains, even then they may be present in appreciable numbers only for a short period of time. However, it would seem that in exceptional cases—exemplified by strain *Cc*—stumpy forms in widely fluctuating numbers may persist for years.

Like the thin trypanosomes, the stumpy forms in *T. evansi* (Fig. 3, *o–v*) are indistinguishable from those in the *Brucei* group. They are typically represented by trypanosomes without a free flagellum (Fig. 3, *o–s*, *v*), but in some (Fig. 3, *t*, *u*) a short one may be present. The posterior end of the body is usually rounded and somewhat swollen (Fig. 3, *o–s*), being 'like the head of a turtle', to use Bruce's graphic expression, while in some specimens this end may resemble a 'hippopotamus head', to use another simile coined by Bruce, and occasionally the posterior end may terminate in a short point (Fig. 3, *u*). Normally the nucleus of stumpy forms lies somewhere in the middle of the body, but in strain *Cc* the nucleus in many individuals was displaced posteriorly, occupying a position near the starting-point of the flagellum (Fig. 3, *w*, *x*): such trypanosomes are identical to the posteronuclear (='posterior-nuclear') forms encountered in members of the *Brucei* group. As regards the kinetoplast, it is terminal or subterminal in position.

In the account given above, I have restricted myself to the qualitative characters of the different forms in *T. evansi*. As regards the quantitative characters, those relating to substrains *Cc* and *Nb* are shown in Fig. 1 and Table 3, but the mensural data for all the available strains, which have been treated statistically, will be dealt with fully in section IV (see p. 155).

(*e*) *Significance*

We now turn to the nature and significance of polymorphism in *T. evansi*. In the case of the *Brucei* group, a number of hypotheses have been advanced to explain this phenomen. In view of the close similarity of polymorphism in members of this group and in *T. evansi*, it will be necessary first to consider these views, and then discuss their bearing on the conditions observed in the last-named species.

Robertson (1912, 1913) interpreted polymorphism in *T. gambiense* as stages in growth from the stumpy forms, through intermediate ones, to the slender forms. The latter represent the reproductive stage, which divide, giving rise again to stumpy forms. These were regarded as the 'adult' stage, which does not multiply

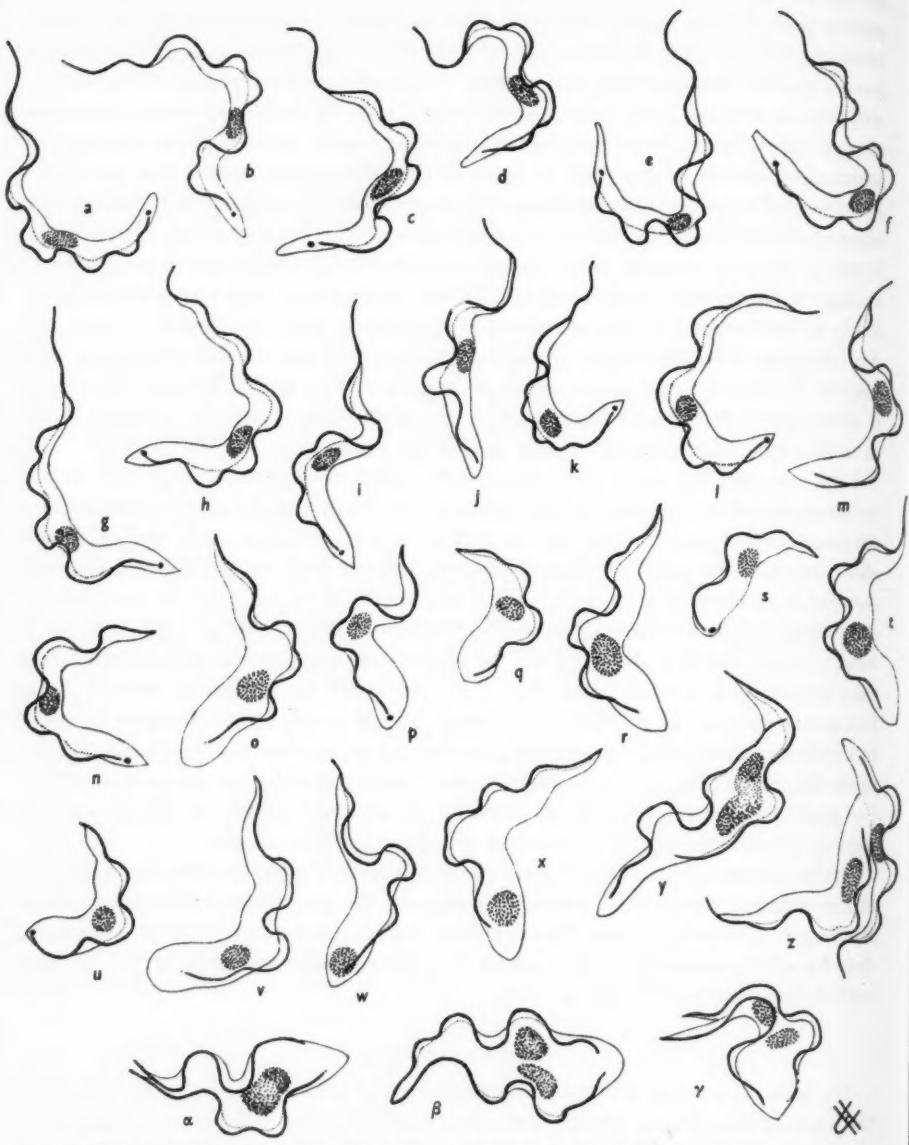

Fig. 3. *T. evansi*. *a–f*: slender forms (*A*, *J*, *K*, *C*, *E*, *Q*); *g*, *h*: transitional forms between slender and intermediate (*L*); *i–l*: intermediate forms (*A*, *B*, *J*, *W*); *m*, *n*: transitional forms between intermediate and stumpy (*C*, *X*); *o–x*: stumpy forms (*C*, *AA*, *C*, *C*, *N*)—free flagellum absent in *o–s*, present in *t*, *u*; *v*: 'hippo-headed' form; *w*, *x*: posteronuclear forms; *y–z*: two stages of unequal division of intermediate forms resulting in stumpy + intermediate progeny; *α*, *β*, *γ*: three stages of division of stumpy forms. Capital letters in brackets denote strains (see Table 1) to which the trypanosomes belong. (Camera lucida drawings, at × 2000.)

but is responsible for carrying on the cycle in the insect-vector. However, Duke (1930, 1935) doubted whether the stumpy forms represented the infective stage, though he believed that transmissibility of trypanosomes of the *Brucei* group to the tsetse-fly depended on the existence in the blood infection of 'positive' and 'negative' phases. A different view was expressed by Reichenow (1921), who suggested that the type of trypanosome found in an infection reflected the degree of virulence of the strain, thin forms predominating when the course of the disease was acute, and stumpy forms when it was mild. A revolutionary approach to this problem was made by Fairbairn & Culwick (1946). They quote Vanderplank's observation (from a personal communication) that the slender and stumpy forms in *T. rhodesiense* differ in the number of nuclear chromosomes, but state that they themselves could not detect this difference. On the other hand, these authors claim to have demonstrated, by statistical methods and from the appearance of the trypanosomes in blood films, the existence of a form of conjugation in this species. They believed that sexual union takes place between two slender forms, giving rise to stumpy and intermediate ones, while syngamy between these two forms produces slender ones, which may either divide or re-enter into syngamy. Though these authors observed and figured stumpy forms having the appearance which 'might suggest binary fission', they regarded them as two conjugants, united 'head-to-head'. They also believed that, in cyclically transmitted species, syngamy, being associated with a change of environment, is a process which enables the trypanosomes to adapt themselves to new conditions, and therefore has a survival value for the strain. However, when these trypanosomes are transmitted from one mammal to another by syringe passages, this necessity does not arise, and syngamy is dispensed with, with the result that, in strains of the *Brucei* group maintained in the laboratory, stumpy forms disappear and the population becomes monomorphic, being represented only by thin forms, which are propagated by 'vegetative division'.

Since polymorphism in *T. evansi*—especially as manifested in strain *Cc*—is in every respect comparable to that in the *Brucei* group, the hypotheses regarding this phenomenon can be considered not only on their own merits but also in the light of my observations on the Surra trypanosomes. In the first place, there is no experimental evidence to support the claim that the stumpy forms in the *Brucei* group represent the stage infective to the insect-vector. In fact, all my attempts to transmit *T. evansi* to tsetse-flies failed, in spite of the presence, at the time of the experiments, of stumpy forms in *Cc*, which was one of the strains used for this purpose (Hoare, 1940*a*). As regards the question of syngamy, however plausible this hypothesis might be from the statistical point of view, it is not supported by any cytological evidence of nuclear changes in the 'conjugants', such as meiosis and exchange of chromosomes, without which the very existence of sexual reproduction in trypanosomes is doubtful. Furthermore, the teleological interpretation of the disappearance of stumpy forms in laboratory strains of the *Brucei* group is in contradiction with the facts observed by me in *T. evansi*. Though this trypanosome has no intermediate host but is transmitted mechanically, both in nature and in the laboratory, and is typically monomorphic, strain *Cc* has retained a high degree of

polymorphism for many years, actually longer than hitherto recorded for any laboratory strains of the *Brucei* group. The loss of polymorphism in such strains is now a well-established fact. It was apparently first mentioned by Bruce (1914) in the case of *T. brucei*, while Yorke (1914) reckoned that this change has taken place in the course of 3 years. Some years ago (1930–4: unpublished) I kept under observation strains of all the three species of this group in guinea-pig passages, and found that (*a*) in *T. gambiense* polymorphism was present only up to 7 months after the strain had been isolated from man, (*b*) in *T. rhodesiense* an appreciable degree of polymorphism was observed for 12 months after isolation, after which stumpy forms occurred only occasionally for another 12 months, and (*c*) in *T. brucei* polymorphism persisted for 30 months after isolation from an infected dog.

Another question which is in need of elucidation, is the absence of division in stumpy forms, postulated by Robertson, on the one hand, and by Fairbairn & Culwick, on the other. Though it is generally agreed that division is usually observed in the thin forms, a number of observers have briefly recorded and depicted, in one or another species of the *Brucei* group, stumpy forms in the process of division. These findings, as well as his own original observations, were dealt with exhaustively by Lavier (1927), who provided convincing evidence, with numerous illustrations, of the reproduction of stumpy forms by binary fission in *T. gambiense*, *T. brucei* and *T. rhodesiense*, the proportion of dividing individuals among these forms reaching 3 % in the last-named species. Lavier has shown that the arrangement of the daughter-elements in dividing stumpy forms is typically bilaterally symmetrical, in contrast to the dividing thin forms, in which they are arranged asymmetrically. This author also demonstrated that posteronuclear forms could only originate by fission of the stumpy forms. In those cases when the daughter nuclei come to lie side by side in the transverse plane, both products of division are ordinary stumpy forms, but when the two nuclei are arranged in the longitudinal plane, one of the daughter-individuals becomes an ordinary stumpy form, the other a posteronuclear one. This interpretation is supported by Lavier's observation that the emergence of crops of posteronuclear forms is always preceded by the appearance in the blood of dividing stumpy forms, the number of which was found to be proportional to that of the posteronuclear forms.

In the strains of *T. evansi* studied by me, a fair number of stumpy forms were encountered in all stages of division and conforming to the pattern described by Lavier, as shown in Fig. 3, α–γ. These dividing forms are indistinguishable from the figures given by Fairbairn & Culwick (1946: pl. XIX, figs. 32, 31), as illustrations of syngamy between stumpy forms in *T. rhodesiense*. Moreover, the high proportion of posteronuclear forms seen by me in *Cc* (up to 60 %) would seem to indicate that division of stumpy forms in this strain was a common phenomenon. It remains to consider other possible factors which might influence the polymorphism of *T. evansi*, such as the rate of multiplication of the trypanosomes and the akinetoplastic condition of strain *Cc*. It would appear that neither of these factors can account for this phenomenon. In the first place, stumpy forms made their appearance both on days when parasitaemia was high and when it was low. Secondly, although the absence of a kinetoplast in trypanosomes was associated

with a high degree of polymorphism in strain *Cc*, which is totally akinetoplastic, there was no such correlation in strain *AA*, in which the maximum number of stumpy forms never exceeded 2 %, in spite of a wide fluctuation in the proportion of akinetoplastic trypanosomes (up to 70 %) observed in it during the first year (cf. Hoare & Bennett, 1939). On the other hand, in *Nb* the proportion of stumpy forms reached 25 %, though akinetoplastic forms were practically absent in this strain.

From the discussion of the conflicting views regarding polymorphism in the *Brucei* group and in *T. evansi*, it is evident that none of them provides a satisfactory explanation of the nature and significance of this phenomenon.

IV. TYPICAL STRAINS

(*a*) *General characteristics*

The observations described in the preceding section have provided conclusive evidence that, in typical strains, the Surra trypanosomes are practically monomorphic, in the sense of being represented almost exclusively by thin (slender and intermediate) forms, whereas stumpy forms are usually absent or present only in negligible numbers (cf. Table 2). Only in exceptional cases some strains (e.g. *Nb*, *Cc*), when examined continuously, may reveal a high degree of polymorphism, persisting for various periods of time. Polymorphism in *T. evansi* is therefore an inconstant feature appearing only sporadically. Although it is generally accepted that all the Surra trypanosomes are morphologically very similar, the published records seem to indicate that strains of different provenance vary in their dimensions, as shown in Table 4. Since such strains were derived from diverse animal hosts of various countries, it might be inferred that strains of different origin are characterized by distinct mensural characters. In fact, both this criterion and the cross-immunity test have been used by a number of authors to separate certain strains from *T. evansi* as distinct species or varieties, the names of which are given on p. 161.

(*b*) *Biometry*

From the inspection of the abundant and varied collection of blood films at my disposal, I was able to satisfy myself that morphologically the thirty populations listed in Table 1 were represented by the same type of thin trypanosomes, and therefore conformed to the typical monomorphic pattern of *T. evansi*. In view of the discrepancy in the mensural data obtained by previous authors and the uncertainty regarding the taxonomic status of various hostal and geographical strains, it was decided to carry out a biometrical study, in order (*a*) to verify the measurements of the trypanosomes in this species, (*b*) to ascertain whether the populations in my collection showed any evidence of variation in dimensions, and, if so, (*c*) to determine the statistical and taxonomic significance of this variation. For this purpose I selected twenty-two populations (strains *A* to *S* in Table 1) of trypanosomes from cases of Surra in six species of animal hosts obtained from fifteen different localities, extending from Morocco to the Philippines. Since the qualitative characters of the trypanosomes in all the strains were indistinguishable, they were

compared on the basis of measurements of the total length of non-dividing individuals (from the posterior end of the body to the tip of the flagellum), by the method described on p. 133. In twenty strains the samples measured consisted of 100 trypanosomes, but in *Cc* and *Nb* the counts were 273 and 229 respectively, representing only the thin forms in these polymorphic samples (cf. Table 2). In all cases the

Table 4. T. evansi: *previous statistical data*

		Measurements of length (μ)				
Hosts	Countries	Range	M	σ	Nos. measured	Authors
1. Camel	A.E. Sudan	20–26	23·1		?	Fry (1911)
2. Camel	A.E. Sudan	17–29	24·0		?	Bruce (1911)
3. Camel	India	18–28	24·0		?	Bruce (1911)
4. Camel	?E. Africa	18–28	24·0		100	Bruce (1911)
5. Lab. animals *ex* camel	Abyssinia	18–34	25·5		400	Duke (1912)
6. Dog *ex* camel	A.E. Sudan	20–29	24·0		?	Fry (1911)
7. Dog *ex* camel	A.E. Sudan	22–26	23·2		?	Fry (1911)
8. Horse	?E. Africa	18–30	25·0		120	Bruce (1911)
9. Horse	India	17–32	23·3		300	Ray (1945)
10. Horse	India	17–30	24·6		300	Ray (1945)
11. Horse	India	17–29	22·9		300	Ray (1945)
12. Horse	Bulgaria	20–27	?		?	Pavlov & Guenev (1939)
13. Dog *ex* horse	Morocco	17–31·5	24·8		270	Barotte (1925)
14. Cattle	India	21–38	28·3		300	Ray (1945)
15. Cattle	India	20–36	28·4		300	Ray (1945)
16. Cattle	India	23–33	27·2		300	Ray (1945)
17. Cattle	India	18–33	25·0		?	Rao & Mudaliar (1934)
18. Cattle	India	19·4–35·3	27·9		80	Lingard (1907)
19. Cattle	Philippines	?	18·4	2·2	?	Manresa & Gonzalez (1935)
20. Buffalo	Philippines	?	19·8	2·0	?	Manresa & Gonzalez (1935)
21. Buffalo	India	?	21·0		100	Mudaliar (1945)
22. Dog	?E. Africa	21–30	25·7		100	Bruce (1911)
23. Dog	?	18–34	24·1		700	Nani & Vergati (1951)
24. Various (total)	Various	18–34	24·5	2·6	820	Bruce (1911) [calculations by Dr J. C. Broom]

trypanosomes were measured in the order in which they appeared in view. The statistical constants calculated (shown in Table 5) were the mean (M) and the standard deviation of the series (σ), as well as twice the standard deviation of the mean ($2\sigma_M$).

The statistical data for the measurements of the total lengths of trypanosomes in the twenty-two strains and substrains of *T. evansi* are represented graphically in Fig. 4, by the method devised by Dice & Leraas (1936), with modifications introduced by Davis (1952). This method is in many ways superior to the conventional distribution curves, for it enables the reader to compare the mensural data of a large series of samples at a glance, and can be reproduced with considerable economy in space and cost. In this chart the range of lengths is shown by horizontal lines

and the means are indicated by cross-bars. On either side of the mean, the limits of the black rectangles correspond to the standard deviation of the series, those of the white rectangles to twice the standard deviation of the mean. According to Dr Trevan, when the white rectangles ($=2\sigma_M$) for two samples just do not overlap, the probability that they are samples of the same population is (*a*) only 0·02, provided the standard deviations are the same and the samples are of the same size (more than thirty individuals), and (*b*) not more than 0·05, if the standard deviation of

Table 5. T. evansi: *statistical constants for measurements of length in twenty-two typical populations*

Strains	Mean lengths (μ)	σ	No. measured	Strains	Mean lengths (μ)	σ	No. measured
A	22·8	2·6	100	*J*	27·4	3·3	100
B	21·1	2·5	100	*K*	26·2	2·5	100
C	22·7	1·6	100	*L*	25·5	2·2	100
Ca	23·7	2·2	100	*M*	20·6	1·7	100
Cb	24·0	2·2	100	*Na*	23·0	2·2	100
Cc	23·2	3·1	273	*Nb*	21·2	2·4	229
D	24·0	2·4	100	*O*	25·5	2·5	100
E	23·7	2·1	100	*P*	23·9	2·5	100
F	23·7	2·5	100	*Q*	25·1	2·5	100
G	24·1	2·6	100	*R*	25·4	2·5	100
H	24·6	2·4	100	*S*	25·0	3·5	100
				Total	23·9	3·0	2502

one is smaller than that of the other, or the samples are of different sizes. Therefore, if—in any set of samples compared—the white rectangles do not overlap, there is reasonable probability that the differences in the measurements are statistically significant, their significance increasing with the distance between these rectangles.

The populations in Fig. 4 are arranged primarily according to the hosts from which the samples were taken, and secondarily—within each host-group—according to the geographical region to which the hosts belonged (cf. Table 1). It is evident from a glance at this chart that the mean lengths of the trypanosomes in the populations under consideration vary considerably, the variation being particularly striking if the samples are compared in the ascending order of their means, viz. *M*, 20·6; *B*, 21·1; *Nb*, 21·2; *C*, 22·7; *A*, 22·8; *Na*, 23·0; *Cc*, 23·2; *Ca*, *E*, *F*, 23·7; *P*, 23·9; *Cb*, *D*, 24·0; *G*, 24·1; *H*, 24·6; *S*, 25·0; *Q*, 25·1; *R*, 25·4; *L*, *O*, 25·5; *K*, 26·2; *J*. 27·4. In this series the means show a fairly regular gradation from 20·6 μ at one end (*M*) to 27·4 μ at the other (*J*), the difference between these extremes, when tested in terms of *t* value, being 18·5, which is statistically so highly significant that the probability of its being due to chance is considerably less than 10^{-11}, i.e. the odds of significance are more than 100 milliard to one. Having demonstrated the range of variation in the mean length of the trypanosomes from all sources, we can compare the strains from (*a*) different hosts, and (*b*) different geographical areas, according to the plan outlined above. Within these groups the samples were also arranged in the ascending order of the means, then each sample was compared with the succeeding one, and finally a comparison was made between the end members of the series. In

the case of cameline strain *C*, samples of its substrains were compared in different combinations. In assessing the significance of the difference between two samples, odds below 20 to one were regarded as statistically not significant, those between 20

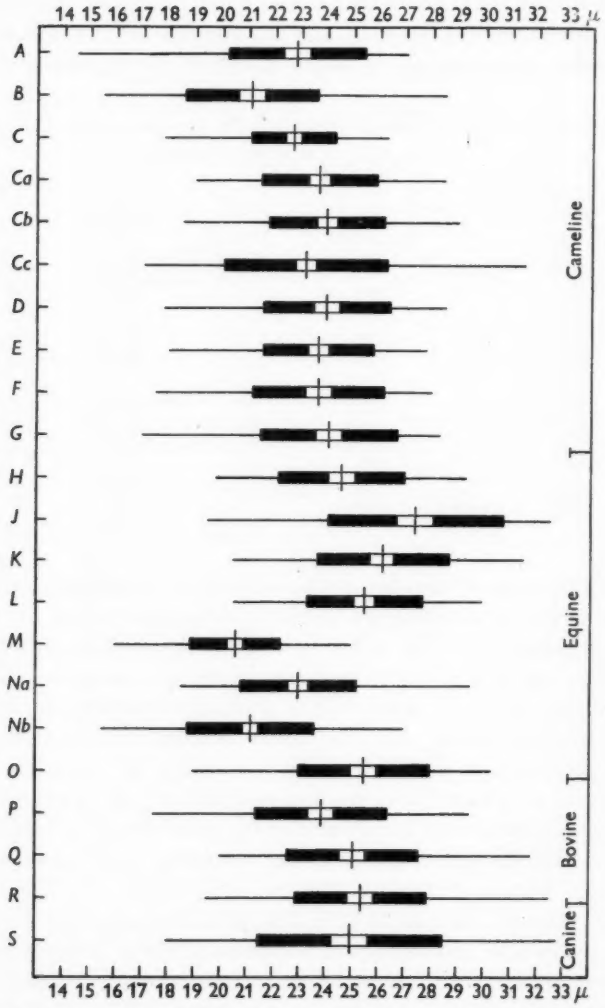

Fig. 4. *T. evansi*. Variation in length of twenty-two populations (*A* to *S*: cf. Table 1). Range of length is shown by horizontal line, means by cross-bars; on either side of the mean, black rectangles represent standard deviation of the series (σ) and white rectangles represent twice the standard deviation of the mean ($2\sigma_M$).

and 100 to one as probably significant, those between 100 and 1000 to one as significant, and those above 1000 to one as highly significant; when the odds exceeded one million to one, they were recorded as $\infty : 1$.

The basis of comparison and the statistical results of this study are shown in Table 6, from which it is seen that the difference between the means of some pairs of cameline strains (nos. 2–9) is statistically not significant, but that of others is highly significant, the odds for no. 1 being as high as 12 million to one, while the difference between the end members of this series (no. 10) is much greater (*ca.* 10 milliard to one). The equine (nos. 17–24) and bovine (nos. 25–27) strains proved to be even more variable. Thus the differences between the equine samples are statistically not significant only in two pairs (nos. 21, 22), whereas in six pairs (nos. 17–20, 23, 24) the difference is significant, the likelihood of such differences arising by chance being from one in a hundred to one in 100 milliard (in no. 24, *M–J*, which also represent the extreme means mentioned above). Similarly, in the bovine strains one pair (no. 26) shows a difference that is not significant, but that of the other (no. 25) is highly significant, while the odds of significance between the end members of this series (no. 27) are as high as 20,000 to one.

The difference between the mean lengths is especially illuminating in the case of substrains derived from one and the same strain, exemplified by the cameline populations *C*, *Ca*, *Cb*, *Cc*, on the one hand, and by the equine populations *Na* and *Nb*, on the other. The pairs of cameline samples (nos. 11–16), which were compared in various combinations, revealed differences that are significant (160:1) in one case (no. 15) and highly significant (2500:1–500,000:1) in two cases (nos. 12, 13). The difference between the two equine substrains (no. 18) is still more marked, the odds of significance being one milliard to one.

We now turn to a comparison of strains from different geographical areas, irrespective of the hosts. With the exception of the equine populations from Bulgaria (no. 28) considered above (= no. 18) the remaining pairs of samples from the Palaearctic region, represented by cameline strains from the Soviet Union, are not significantly different. In the Oriental region, the difference between four pairs of samples (nos. 32–35) is also not significant, but in the others (nos. 36–38) it is significant in various degrees, the odds in one case (no. 38) being 20,000 to one, in another (no. 37) 10 milliard to one. As regards the Ethiopian region, it is represented by cameline strains from the Anglo-Egyptian Sudan and Somaliland (nos. 39–45), most of which have already been dealt with above (see nos. 1–4, 16, 8): to these should be added the difference between the end members of the series (no. 45), the significance of which is extremely high (10 milliard to one).

From the foregoing account it is evident that (*a*) there is considerable variation in the mean length of *T. evansi* belonging to diverse hostal and geographical strains, and (*b*) the difference in the mean length between various samples is statistically significant in almost 50 % of the pairs compared. A precise statistical analysis of the mensural data for these strains is given by Dr Trevan in the Appendix, to which the reader is referred for further information.

(*c*) *Significance of variation*

Since one of the objects of the present biometrical study was to ascertain whether strains of *T. evansi* from diverse hosts and localities can be distinguished by constant quantitative characters, it is necessary to consider the taxonomic value of

Table 6. T. evansi: *statistical significance of differences in mean lengths between strains (cf. Fig. 4 and Table 5)*

(Calculations by Dr J. W. Trevan.)

Basis of comparison			Pairs of strains compared	*t* values	Odds of significance (approximately)
Samples from same host-species	Cameline	Strains from all sources	1. *B–C*	5·4	∞ : 1
			2. *C–A*	0·3	2 : 1
			3. *A–Cc*	1·3	5 : 1
			4. *Cc–Ca*	1·8	16 : 1–20 : 1
			5. *Ca–E*	0	1 : 1
			6. *E–F*	0	1 : 1
			7. *F–Cb*	0·9	3 : 1
			8. *Cb–D*	0	1 : 1
			9. *D–G*	0·3	2 : 1
			10. *B–G**	8·3	∞ : 1
		Substrains of same strain (*C*)	11. *C–Cc*	2·0	20 : 1
			12. *C–Ca*	3·7	2,500 : 1
			13. *C–Cb*	4·8	500,000 : 1
			14. *Cc–Ca*	1·8	16 : 1–20 : 1
			15. *Cc–Cb*	2·8	160 : 1
			16. *Ca–Cb*	1·0	3 : 1
	Equine	Strains from all sources	17. *M–Nb*	2·6	100 : 1
			18. *Nb–Na*	6·6	∞ : 1
			19. *Na–H*	5·9	∞ : 1
			20. *H–L*	2·8	160 : 1
			21. *L–O*	0	1 : 1
			22. *O–K*	2·0	20 : 1
			23. *K–J*	2·9	250 : 1
			24. *M–J**	18·5	∞ : 1
	Bovine		25. *P–Q*	3·7	2,500 : 1
			26. *Q–R*	0·8	3 : 1
			27. *P–R**	4·3	20,000 : 1
Samples from same geographical area	Eurasia	Palaearctic: Bulgaria	28. *Nb–Na*	6·6	∞ : 1
		U.S.S.R.	29. *E–F*	0	1 : 1
			30. *F–G*	1·1	3 : 1
			31. *E–G**	1·2	3 : 1–5 : 1
		Oriental: India, Indo-China, Malaysia	32. *P–H*	2·0	20 : 1
			33. *H–R*	2·3	20 : 1–50 : 1
			34. *R–L*	0·3	2 : 1
			35. *L–K*	2·1	20 : 1
			36. *K–J*	2·9	250 : 1
			37. *P–J**	8·5	∞ : 1
		Indo-China (bovine)	38. *P–R*	4·3	20,000 : 1
	Africa	Ethiopian: East	39. *B–C*	5·4	∞ : 1
			40. *C–A*	0·3	2 : 1
			41. *A–Cc*	1·3	5 : 1
			42. *Cc–Ca*	1·8	16 : 1–20 : 1
			43. *Ca–Cb*	1·0	3 : 1
			44. *Cb–D*	0	1 : 1
			45. *B–D**	8·4	∞ : 1
Samples showing difference of subspecific order			46. *B–K*	11·7	∞ : 1
			47. *K–Nb*	14·4	∞ : 1
			48. *M–H*	13·6	∞ : 1
			24. *M–J*	18·5	∞ : 1

* Pairs marked with an asterisk represent the end series of the group.

the differences established between these strains statistically. Though there are no universally recognized quantitative criteria for determining taxa, it is usual to separate allied groups of animals as species when their structural characters do not intergrade, and as subspecies, or minor units, when these characters intergrade. Theoretically, therefore, in the case of groups differing in the frequency distribution of the length of the organisms, species will be represented by curves separated by a gap, whereas in subspecies the curves will overlap.

As regards the magnitude of the difference between the mean lengths of these taxa, the following examples can serve as illustrations. The differentiation of two species on biometrical grounds was established, in the case of *Trypanosoma uniforme* and *T. vivax*, by Hoare & Broom (1938). They demonstrated that each of these species consisted of a set of strains, the mean lengths of which differed significantly—in a statistical sense—from one another in many cases. However, within each species the mean lengths of the strains showed a relatively continuous variation, whereas there was a marked gap between the highest mean of the shorter species and the lowest mean of the longer species. In connexion with the present study, the means of these two 'boundary' strains were compared, and the result was as follows:

$$\left.\begin{array}{lll} T.\ uniforme\ (\mathrm{L}) & M = 16{\cdot}1\,\mu & \sigma = 1{\cdot}3 \\ T.\ vivax\ (\mathrm{F}) & 21{\cdot}0\,\mu & 1{\cdot}0 \end{array}\right\} t = 30,\ \text{odds}\ \infty : 1.$$

From these figures it is evident that the statistical significance of the difference between these species is considerably greater than that observed in any of the pairs of samples of *T. evansi* compared in the present study. As regards the differentiation of subspecies on the basis of measurements of length, Hubbs & Perlmutter (1942) have shown that in cases when the black rectangles (representing standard deviations by the Dice-Leraas method) neither overlap nor are separated—indicating that 84 % of the specimens in such samples would be separable—the difference between the corresponding populations can be regarded as being of subspecific order. In the case of *T. evansi*, examples of pairs in which the black rectangles are contiguous are provided by *B–K*, *K–Nb* and *M–H* (Fig. 4), the statistical significance of the differences between which is shown in Table 6 (nos. 46, 47, 48 respectively). If these statistical data were used as criteria for the determination of the taxonomic status of these strains, the two samples in each of the above pairs would have to be assigned to distinct subspecies, while samples separated by gaps between the black rectangles (e.g. *M–J* = no. 24 in Table 6) might even be regarded as species. However, such an interpretation is untenable even on statistical grounds, for, as has been demonstrated above in the case of *T. uniforme* and *T. vivax*, the strains referable to these species fall into two corresponding groups, whereas in *T. evansi* there is no real break in variation between one strain and another, when they are arranged in the ascending order of their mean lengths; therefore, they should be classed in one group.

There is likewise no justification for separating any of these strains into infraspecific systematic units, because—as seen from Fig. 4 and Table 6—on the one hand, there is no significant difference, as regards mean length, between a number

of unrelated strains from diverse hosts and localities (e.g. $B = Nb$, $C = A$, $Na = Cc$, $Ca = E = F$, $P = Cb = D = G$, $S = Q$, $L = R = O$), while on the other hand, there is a significant difference (*a*) between strains from the same host species (cf. Table 6, nos. 1–10, 17–27) or the same geographical area (cf. Table 6, nos. 28–45), and even (*c*) between substrains of one and the same strain (cf. Table 6, nos. 11–16, 18). Hence it follows that, in spite of the wide discrepancies in the mean length among the twenty-two strains in question, they should be regarded as belonging to a

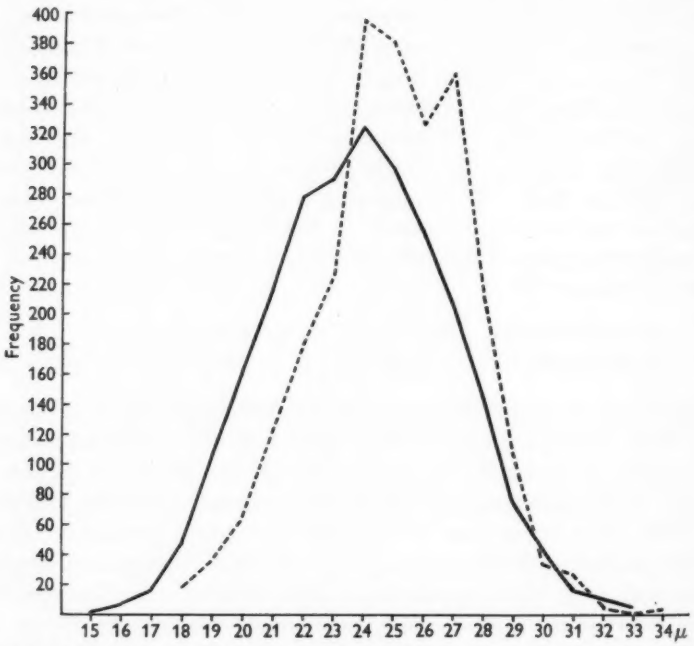

Fig. 5. *T. evansi*. Two length distributions of *ca.* 2500 trypanosomes each: (*a*) ———, total for twenty-two populations in present work (*A* to *S*: cf. Table 1 and Figs. 4, 6); (*b*) – – – –, total for 6 populations in Bruce (1911).

single species, *T. evansi*. As regards the underlying cause of observed variation, it may be due to heterogeneity of the samples, produced by some physiological factors, such as crops of trypanosomes of different ages (this question is fully dealt with by Dr Trevan in the Appendix). Whatever the nature of this variation may be, the essential unity of the strains of Surra trypanosomes—already discernible in the gradation of the mean values—is clearly brought out when the measurements of length for the twenty-two samples are pooled and plotted. Thus the curve for the grand total of 2500 counts (Fig. 5: full line) shows a normal length distribution, with a range from 15 to 33μ, and mean = 24μ, while probit analysis (by Dr Trevan) shows practically a straight line (Fig. 6). The only other available figures for *T. evansi* on a comparable scale were provided by Bruce (1911), who measured a total of 820 trypanosomes belonging to strains from six animal species, the statistical

constants of which were kindly calculated by Dr J. C. Broom (cf. Table 4, no. 24). In fig. 5 the length distribution of Bruce's measurements (corrected to 2500) is indicated by the interrupted curve, which also shows a normal distribution, with a range from 18 to 34 μ, and mean = 24 μ, thus practically coinciding with and lending support to the results obtained by me.

From these figures it can therefore be concluded that, when the sum total of a large number of measurements is considered, the variations between the component populations are smoothed out, with the result that an approximately true

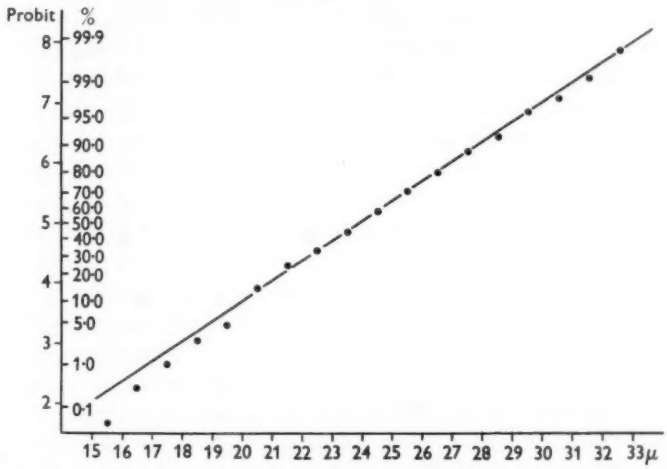

Fig. 6. *T. evansi*. Probit values for the total (= 2502) measurements of length in twenty-two populations (cf. Table 5 and Figs. 4, 5); dots indicate observed percentages, the line corresponds to $\sigma = 3$. (From plots by J. W. Trevan.)

mean characteristic of the group as a whole is arrived at. As regards the variation in length observed in the diverse samples studied, the degree of the statistical significance of the difference between them does not provide any criterion for distinguishing the corresponding strains taxonomically. These results serve to emphasize once more that the statistical significance of mensural data cannot always be interpreted in terms of taxonomic and biological significance. Results comparable in every respect to those recorded for *T. evansi* in the present investigation were also obtained by Davis (1952) in a biometrical study of *T. lewisi* from various Californian rodents.

(*d*) *Clines*

Although the diverse hostal and geographical strains of *T. evansi* cannot be separated on the basis of mensural characters, in the case of the equine strains there is a more or less uniform gradation in the length of trypanosomes over the area of distribution of the host. This is illustrated in Fig. 7, in which the mean lengths of the equine strains, measured by myself (shown by dots) and by other authors (shown by circles; also see Table 4), are plotted on a spot diagram against the

approximate longitude of the country of origin. From this diagram it is evident that there is a tendency for the means to increase with longitude. According to Dr Trevan, who has made the necessary calculations, this gradation is significant, especially in the strains measured by me. In these the coefficient of correlation is as high as 0·869, which would be obtained by chance alone from the eight observations less than once in a hundred trials. Therefore these figures indicate a real tendency for the length of the trypanosomes in the equine host to increase from West to

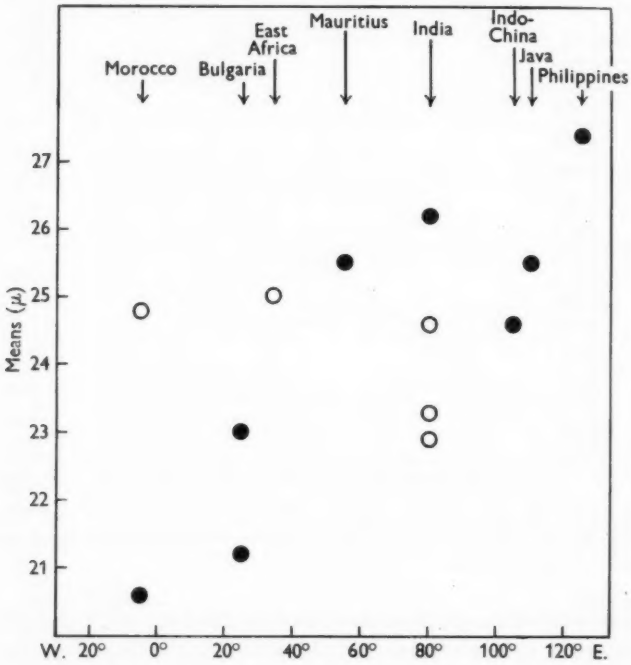

Fig. 7. *T. evansi.* Clines (=clinodemes) showing continuous gradation in mean lengths of strains over the geographical range of distribution of the equine hosts: dots indicate present figures, circles show figures from literature. (From plots by J. W. Trevan.)

East. Since this variation is continuous, there is no grouping of mean lengths to indicate taxonomically separable units. As regards the few figures for equine strains recorded by other authors (cf. Table 4), they do not show this trend. If they are included with the strains studied by me, the correlation ratio falls but is still significant. However, since these measurements were made by different workers, slight modifications in measuring technique may have introduced a bias.

The type of variation observed in the equine strains of *T. evansi* corresponds to the concept of 'clines' (Huxley, 1938) or 'clinodemes' (Gilmour & Heslop-Harrison, 1954), which are common among populations of free-living organisms and are usually determined by purely geographical factors, such as climate. Since the environmental conditions to which the trypanosomes are exposed within the

equine host in various geographical regions are presumably similar, and independent of external influences, the observed length-gradient is probably determined by some physiological or other—unknown—factors. There is some indication that the progressive increase in the length of *T. evansi* from West to East might be correlated with the virulence of the equine strains in different geographical areas. From a perusal of the relevant literature and from personal communications, the position would appear to be as follows. In North Africa the infection in horses usually runs a mild chronic course, while in the Anglo-Egyptian Sudan (Hoare & Bennett, 1939), Somaliland and northern Kenya (communicated by Mr E. F. Peck) equines are either resistant or the infection in them is inapparent. Further to the East the virulence of equine trypanosomes seems to increase, for in the Middle East (Syria, Palestine) the onset of the disease is subacute, while in Soviet Middle Asia and India it is acute, though in both these regions the infection eventually becomes chronic. However, in the extreme eastern area of distribution of equine Surra (Indo-China, Java, Philippines) the course of the disease is acute and fatal, and epizootic outbreaks are common. Exceptions are provided by Bulgaria (*ca.* 25° E.) and Mauritius (*ca.* 55° E.), where the disease in horses runs an acute course, but since in the former the infection was probably introduced from Asia Minor, and in the latter it was definitely imported from India, equine Surra in these localities conforms to the pattern of the countries of origin.

It should be emphasized again that, although the transition from non-virulent strains of equine Surra in the West to virulent ones in the East shows some correlation with a progressive increase in the mean length of the corresponding trypanosomes, this variation is gradual, without any break between the strains. This is in contrast with *T. vivax*, in which, according to Fairbairn (1953), East and West African bovine strains, which differ in virulence, are separable by the mean length of the trypanosomes in these areas.

(*e*) *Diagnostic mensural characters*

Having demonstrated that the variation in the mean length of diverse strains of the Surra trypanosomes affords no criterion for regarding them as independent taxonomic units, we are now in a position to supplement the qualitative characteristics of *T. evansi* (described in section III) by the quantitative characters of this species. In the foregoing discussion it was shown that the pooled measurements of length of all the strains studied by myself and by Bruce (1911), comprising a total of 3320 trypanosomes, provide reasonably accurate mensural criteria for the diagnosis of this species as a whole. Therefore, if the inconstant occurrence in this species of stumpy forms is disregarded, it can be concluded that typically *T. evansi* measures from 15 to 34μ in length, with a mean of 24μ (see Fig. 5). When this species exhibits polymorphism, the mean measurements of the component forms are as follows: stumpy 16·8–19·6μ, intermediate 19·5–20·7μ, slender 23·0–24·9μ (in *Nb* and *Cc* respectively: Table 3).

It remains to consider the measurements of *T. evansi* given by previous authors —other than Bruce (1911)—and their bearing upon the results obtained in the

present study. From Table 4 it is seen that, with a few exceptions, both the lengths and the means recorded by other workers fall within the range of measurements given for the strains studied by me, and show a comparable degree of variation (cf. Table 5 and Fig. 4), which lends itself to a similar interpretation. Results diverging from mine were reported by Ray (1945) in strains from Indian cattle, in two of which the upper limits of the range and the means are higher than in any of my strains (Table 4, nos. 14, 15), and by Manresa & Gonzalez (1935) for Philippine bovine strains, in which the means were lower than any of mine (Table 4, nos. 19, 20). Ray's figures seem to point to a wider variation than that observed by me, but since his figures for Indian equine strains (Table 4, nos. 9–11) also differ considerably from mine relating to a strain of the same origin (*K* in Fig. 4), the discrepancy in our results might actually be due to some difference in the measuring technique. As regards the Philippine strains, in the absence of any data for the range of lengths and the number of trypanosomes measured, the cause of the difference is not clear. The discrepancy in the figures for the trypanosomes of buffaloes is especially puzzling, for the means in Philippine and Indian strains (Table 4, nos. 20, 21) were 19·8 and 21·0 μ respectively, whereas in the Indo-Chinese strain *R*, measured by me, it was 25·4 μ (cf. Table 5). On account of the alleged small size of the buffalo trypanosomes Mudaliar (1945) proposed for them the name *T. evansi* var. *rayi*, but, as shown in Table 5, its mean length (21·0 μ) is well within the range of means of the strains studied by me.

V. SYSTEMATIC POSITION

(*a*) *Affinities*

In the preceding sections the close morphological similarity between *T. evansi* and members of the *Brucei* group has been stressed repeatedly. Thus, the slender and intermediate forms characteristic of typical 'monomorphic' strains, and the stumpy forms occurring sporadically in polymorphic samples of *T. evansi*, are identical to the corresponding forms of *T. brucei*, *T. rhodesiense* and *T. gambiense*. In fact these four species are morphologically indistinguishable, so that the figures depicting *T. evansi* (Fig. 3) can serve equally well to illustrate the structure of these species of the *Brucei* group.

Having established the similarity of the qualitative characters of *T. evansi* to those in the *Brucei* group, we turn to a comparison of the quantitative characters of the trypanosomes in question. For this purpose a selection was made from the literature of those figures for the *Brucei* group which provided the fullest statistical data. The majority of these were based on strains recently studied by Fairbairn & Culwick (1946, 1949) and Culwick & Willett (1947), or on measurements of earlier authors (especially Robertson, Bruce *et al.*) revised by Culwick & Willett (1947), while in a number of cases the missing statistical constants were calculated by Dr J. C. Broom. The resulting measurements for *T. brucei*, *T. rhodesiense* and *T. gambiense*, with references to sources and other particulars, are given in Table 7. Since the measurements of most of the strains of the *Brucei* group pertain to polymorphic populations, the lengths of the stumpy forms, on the one hand, and

of the two thin forms combined, on the other, are given separately in the table, in order to facilitate comparison with either polymorphic or monomorphic populations of *T. evansi*. In the latter case only the thin (slender + intermediate) forms of the *Brucei* group, corresponding to the forms encountered in typical strains of *T. evansi*, need be taken into consideration.

It will be recalled that the overall range of lengths of the trypanosomes in the strains of *T. evansi*, measured by Bruce and by myself, was found to be from 15 to 34μ (Figs. 4, 5), whereas strains measured by other workers ranged from 17 to 38μ, (Table 4). From Table 7 it is evident that, while the lower limit of lengths for the

Table 7. *Measurements of trypanosomes in* Brucei *group*

				Length (μ)			
				Stumpy forms		Thin forms	
Species	Hosts	Origin	Authors	Range	*M*	Range	*M*
T. brucei	1. Monkeys	Uganda	Robertson (1913)	11–22	16·6	14–36	
	2. Various	Zululand	Bruce *et al.* (1915*a*)	11–27	20·0	18–35	
	3. Rats	Tanganyika	Culwick & Willett (1947: *in litt.*)	14–27	19·5	15–39	27·0
	4. Rats	Kenya	Culwick & Willett (1947: *in litt.*)	11–23		15–35	
T. rhodesiense	5. Man	Nyasaland	Bruce *et al.* (1913*a*)	13–25	19·4	16–34	—
	6. Rats	Nyasaland	Bruce *et al.* (1915*a*)	—	—	18–36	28·6
	7. Dog	Nyasaland	Bruce *et al.* (1915*a*)	—	—	20·37	28·4
	8. Rat	Tanganyika	Fairbairn & Culwick (1946)	12–25	17·5	17–42	29·0
	9. Rat	Tanganyika	Culwick & Willett (1947: *in litt.*)	12–27	19·0	16–39	—
	10. Various	E. Africa	Fairbairn & Culwick (1949)	—	—	—	26·9
T. gambiense	11. Monkeys	Uganda	Robertson (1912)	12–26	19·8	20–34	—
	12. Rats	Tanganyika	Bruce *et al.* (1915*b*)	14–27	20·6	21–35	—
	13. Guinea-pig	Belg. Congo	Fairbairn & Culwick (1949)	—	20·3	—	27·2

Notes to Table 7

1. Calculations by Culwick & Willett (1947: *in litt.*).
2. Calculations by Culwick & Willett (*loc. cit.*) based on strain XXV.
3, 4. Mean for both together calculated by Dr J. C. Broom from data in Fairbairn & Culwick (1949): no. 3 is strain 'Shinyanga I' (see Fig. 2).
5. Calculations by Culwick & Willett (*loc. cit.*) based on table XVII.
6. 'Monomorphic' strain: table LXI.
7. 'Monomorphic strain': table LXVII.
9. Tinde strain.
10. Range not given, mean calculated by Dr J. C. Broom.
11, 12. Calculations by Culwick & Willett (*loc. cit.*).
13. Range not given, mean calculated by Dr J. C. Broom.

thin forms in the *Brucei* group lies well within the range of *T. evansi*, the upper limit of the range is somewhat higher. It coincides with that of *T. evansi* in most of the strains (Table 7, nos. 1, 2, 4, 5–7, 11, 12), but in one strain of *T. brucei* (Table 7, no. 3) and in two of *T. rhodesiense* (Table 7, nos. 8, 9) the lengths (39–42μ) exceed the upper limit in *T. evansi* (viz. 38μ; Table 4, no. 14). However, the mean

lengths of the thin forms in the *Brucei* group (range 26·9–29·0μ) are higher than in the great majority of the strains of *T. evansi* studied by me, in only one of which (*J*) has the mean (27·4μ) a comparable value, though the means recorded for *T. evansi* by Ray (1945) were almost as high, viz. 28·3–28·4μ (Table 4, nos. 14, 15). As stated above (p. 156), there is some doubt about the validity of Ray's figures, but even if they are correct it would seem that the means for the three species of the *Brucei* group are, on the whole, higher than in most of the strains of *T. evansi* measured by me and by previous workers. Since the number of trypanosomes measured in the great majority of the strains of *T. evansi* did not exceed 300 (cf. Tables 4 and 5), whereas most of the figures for the *Brucei* group were based on upwards of 1000 measurements, the difference between the two sets can be demonstrated even better if the mean for the pooled lengths of all the strains of *T. evansi*, viz. 24μ (Fig. 5), is compared with the range of means for the *Brucei* group, viz. 26·9–29·0μ (Table 7). It is thus seen that the mean lengths provide the only quantitative criterion for distinguishing typical strains of *T. evansi* from the thin forms in the *Brucei* group.

As regards the stumpy forms in polymorphic samples of *T. evansi*, which measure from 16 to 24μ ($M = 19{\cdot}6\mu$) in *Cc* and from 12 to 21μ ($M = 16{\cdot}8\mu$) in *Nb*, they are well within the range of the length of these forms in species of the *Brucei* group (cf. Table 7). Likewise there is no appreciable difference between the means of the total lengths of all the three forms in the polymorphic samples of *T. evansi* (Table 3), which are 22·0μ (*Cc*) and 19·4μ (*Nb*), and those of species of the *Brucei* group, which vary as follows, according to the curves provided by Mr Culwick and Dr Willett: 17–20μ in *T. brucei*, 19–20μ in *T. rhodesiense*, and 20–21μ in *T. gambiense*. It has also been shown before that the polymorphism in samples *Cc* and *Nb* of *T. evansi* conforms to the pattern established by the Tanganyika workers for the three species of the *Brucei* group (cf. Figs. 1, 2).

The general resemblance between *T. evansi* and members of the *Brucei* group has been noted by a number of previous workers, but, as far as I am aware, Bruce (1911) was the only one to compare their dimensions. From his curves (*loc. cit.*, chart 4) for the length distributions of *T. evansi* and *T. brucei* (18–34 and 13–35μ respectively) he concluded that 'these curves are very unlike', and that therefore 'there ought...to be no difficulty in separating these two species by this means alone'; thus, if it is found that the range of lengths 'lies mostly between 18 and 30 and not between 13 and 35, the diagnosis would be *Trypanosoma evansi*'. But further on he pointed out that, since the means obtained by him in two sets of measurements for each species—viz. 23·6, 23·4μ for *T. brucei* and 24·9, 24·3μ for *T. evansi*—were similar, 'it would, therefore, not be possible to separate these two species by [mean] length alone'. 'The difference between the two is striking', according to Bruce, only if the length distributions or ranges are compared. However, if Bruce's figures are considered in the light of the data discussed by me above, it will be seen that his deduction is misleading. Thus the difference in the ranges—noticeable only at the lower limit—is due to the fact that he compared monomorphic *T. evansi* with polymorphic *T. brucei*, whereas, if the length distribution of the stumpy forms in his figures for *T. brucei* is excluded, it will be evident (cf. Table 7) that Bruce's measurements for the thin forms of both species are very similar.

Neither is there any appreciable difference between the full range of Bruce's *T. brucei* and that of polymorphic samples of *T. evansi*, exemplified by my strains *Cc* and *Nb* (Fig. 1). If these facts are taken into consideration, it will be evident that the representatives of these two species measured by Bruce were practically indistinguishable, in contrast to the results obtained in the present investigation.

Among the morphological characteristics of *T. evansi*, the kinetoplast should also be taken into consideration. In a previous paper (Hoare, 1938) I have demonstrated that the appearance and position of the kinetoplast provide a supplementary criterion for the differentiation of various groups of mammalian trypanosomes. This character proved to be indistinguishable in *T. evansi*, *T. brucei*, *T. rhodesiense* and *T. gambiense*, in all of which the kinetoplast is small (*ca.* 0·6μ), usually rod-shaped, and typically occupies a subterminal or marginal position in the body. In these four species there is also a marked tendency to produce mutant individuals devoid of a kinetoplast, which reaches a maximum in *T. evansi*, giving rise to permanently akinetoplastic strains (Hoare, 1954*a*).

In addition to the morphological characters, all these four species have similar physiological characteristics. Thus, according to von Brand (1951), their metabolism (carbohydrate and oxygen consumption, sensitivity to cyanide and to CH-inhibitors) has the same pattern, and this similarity is reflected in a comparable response of these trypanosomes to the action of specific drugs. In fact, the common physiological and chemotherapeutic characteristics of these four species unite them in a distinct group separable in this respect from all the other mammalian trypanosomes. According to Dr H. E. Hornby (personal communication), both the symptoms and lesions in infections caused by *T. evansi* and *T. brucei* are also alike, and furthermore, since the complement-fixation test does not differentiate these two species, they apparently possess common antigens. Thus, the close affinity between *T. evansi* and members of the *Brucei* group is revealed in their morphology, physiology, antigenic constitution, clinical symptoms and response to drug treatment.

(*b*) *Classification and nomenclature*

In the past, views regarding the systematic position of *T. evansi* were influenced by its monomorphism, which was the only known morphological feature distinguishing it from species of the *Brucei* group. However, there was a good deal of controversy concerning the exact relationship of *T. evansi* to this group. Thus, Bruce (1914, 1915) and Lavier (1933, 1942–3) thought that the occasional presence of stumpy forms in *T. evansi* entitled it to a place within the *Brucei* group, while Duke (1923) proposed to retain it there as an 'appendage'. Other authors (e.g. Yorke & Blacklock, 1914; Wenyon, 1926; Hoare & Coutelen, 1933) disregarded the inconstant occurrence of negligible numbers of stumpy forms in *T. evansi*, and classified it outside the polymorphic *Brucei* group as a monomorphic species. Since it was generally agreed that *T. evansi* was to all intents and purposes monomorphic, this divergence of opinion resolves itself into the importance which different authors attached to the transient occurrence of stumpy forms in some strains, the maximum proportion of which, recorded at that time, did not exceed

0·05 % (see p. 135). In the state of knowledge prevailing in those days, there was some justification for either point of view. However, my discovery in *T. evansi* of a substantial degree of polymorphism, occurring sporadically or persisting for long periods of time, has completely changed the situation. There can now be no doubt whatever that the affinity of *T. evansi* with the *Brucei* group is much closer than has previously been suspected, and that this species is, therefore, entitled by right to a position alongside *T. brucei*, *T. rhodesiense* and *T. gambiense*, in which it had been formerly placed by Bruce and Lavier on grounds which were still debatable.

The classification of *T. evansi* on these lines had already been introduced by me previously (Hoare, 1948*b*, 1949, 1952), but the exact status of this species and its relation to other members of the *Brucei* group were not defined in these publications. Originally this group comprised only species exhibiting constant polymorphism, viz. *T. brucei*, *T. rhodesiense* and *T. gambiense*. Later, when I added to these species *T. evansi* and related species (*T. equinum* and *T. equiperdum*), it became known as the *Brucei-Evansi* group (Hoare, 1949). However, at present this group stands in need of further revision, owing to the recent re-discovery of *T. suis* (Peel & Chardome, 1954), which has a life cycle like that in the polymorphic species, and should therefore be classified in the same group (cf. Hoare, 1954*b*). Since the designation '*Brucei-Evansi*' is no longer representative of its composition, and as the groups in which the mammalian species of the genus *Trypanosoma* are arranged have no taxonomic status, and are therefore not governed by priority, I propose to restore the name '*Brucei* group', dividing it further into three subgroups: *Suis*, *Brucei*, *Evansi*. The revised diagnostic key to this group—which replaces that given in my *Handbook of Medical Protozoology* (Hoare, 1949), but conforms to its general plan—is given below.

Differential diagnosis of the *Brucei* group

Group IV. *Brucei*

Free flagellum: present or absent. Kinetoplast: small (absent in 13). Undulating membrane conspicuous

Subgroups

- Monomorphic
  - (i) *Suis*
    - Stumpy forms with short free flagellum
    - Tsetse: Development in midgut and salivary glands
    - 8. *T. suis*
- Polymorphic
  - (ii) *Brucei*
    - Polymorphism constant
    - Thin forms with free flagellum and stumpy forms without, or with short one, always present
    - Tsetse: Development in midgut and salivary glands
    - 9. *T. brucei*
    - 10. *T. rhodesiense*
    - 11. *T. gambiense*
  - (iii) *Evansi*
    - Polymorphism inconstant
    - Thin forms with free flagellum always present; stumpy forms without, or with short one, rare or sporadic
    - Intermediate host absent
    - Transmission
      - Mechanical
        - Kinetoplast
          - Present
            - 12. *T. evansi*
          - Absent
            - 13. *T. equinum*
      - Contact
        - 14. *T. equiperdum*

We now turn to the nomenclature of *T. evansi*. Since the New World strains have not been specially studied by me—though they were dealt with briefly in another paper (Hoare, 1950)—I shall restrict myself here to Old World strains. It has already been mentioned in the Introduction that distinct names—specific and varietal—have been attached to diverse strains on the basis of serological, hostal, clinical and, in some cases, morphological differences. These claims have been duly considered in other parts of this paper, and it was concluded that there are no grounds for recognizing more than one species, *T. evansi*. The list that follows contains, apart from the original name given by Steel, only synonyms within the genus *Trypanosoma*. These are numbered consecutively, in order to facilitate reference to the accompanying comments, which are based on a perusal of the original literature.

Trypanosoma evansi (Steel, 1885) Balbiani, 1888

Synonyms

1. *Spirochaete evansi* Steel, 1885.
2. *Trypanosoma* [*evansi* (Steel, 1885)] Balbiani, 1888.
3. *T. evansi* var. *mborii* Laveran, 1903.
4. *T. soudanense* Laveran, 1907.
5. *T. elephantis* Bruce *et al.*, 1909.
6. *T. annamense* Laveran, 1911.
7. *T. soudanense* var. *berberum* Sergent, Sergent & Lhéritier, 1912 (*nec* '*T. berberum* Sergent & Sergent, 1904' *auctorum*).
8. *T. cameli* Pricolo & Ferraro, 1914 (*nec* '*T. dromedarii* Pricolo, 1912' *auctorum*).
9. *T. marocanum* Sergent, Lhéritier & Belleval, 1915.
10. *T. ninae kohl-yakimov* Yakimoff, 1921 (*non* 1918).
11. 'Trypanosoma Su-auru', Emelin & Zeiss, 1928*a* [*non* '*T. su-auru* Ilovajskij (=Ilowaisky) *fide* Emelin & Zeiss, 1928*b*, *nec* '*T. su-auru* Emelin & Zeiss 1928' *fide* Yakimoff, 1931].
12. *T. marocanum* var. *cameli* Nattan-Larrier & Noyer, 1931.
13. *T. aegyptum* Nattan-Larrier & Noyer, 1932.
14. *T. evansi* var. *su-auru* Zeiss, 1937 (*nec* Fedorow, 1912).
15. ?*T. kirdanii* Mahomed, 1939.
16. *T. evansi* var. *rayi* Mudaliar, 1945.

Comments on nomenclature

2. This amendment is usually attributed to Crookshank (1886), but there is no evidence in his paper to support this claim. As far as I was able to ascertain, Balbiani (1888) was actually the first to refer the flagellate causing Indian Surra to the genus *Trypanosoma*, which he misspelt '*Tripanosoma*'.

3, 4, 6. *T. evansi* var. *mborii*, *T. soudanense* and *T. annamense* were created by Laveran on the basis of cross-immunity (see p. 131).

5. From Bruce's description it is evident that *T. elephantis* is indistinguishable from *T. evansi*.

7. *T. soudanense* var. *berberum* was separated by the cross-immunity test, while the name '*T. berberum*', though commonly quoted in the literature, has never been proposed by the Sergents, and is therefore *nomen nudum*.

8. *T. cameli* was thus named from its host, whereas the name '*T. dromedarii*', attributed to Pricolo (1912), does not appear in the original paper, and is therefore *nomen nudum*.

9, 10. Both *T. marocanum* and *T. ninae kohl-yakimov* were separated by the cross-immunity test; for the last-named species Yakimoff (1931) erroneously gives the year 1918.

11. 'Trypanosoma Su-auru'—erroneously quoted as a binomial combination—was used merely as a descriptive name for strains distinguished from *T. evansi* on clinical grounds.

12, 13. *T. marocanum* var. *cameli* and *T. aegyptum* were separated partly by the cross-immunity test, and partly morphologically.

14. Though the name *T. evansi* var. *su-auru* was actually proposed by Zeiss (1937) himself, he erroneously credited it to Fedorow.

15. *T. kirdanii* was the name given to a trypanosome resembling *T. evansi*, which was seen in a Sumatra tiger kept in Cairo Zoological Gardens.

16. *T. evansi* var. *rayi* was separated on account of its short length (but see p. 156).

Infraspecific groups. In the present investigation it has been demonstrated conclusively that the diverse geographical and hostal strains of Surra trypanosomes cannot be separated from *T. evansi* either on morphological or on biometrical grounds, in spite of the statistical significance of the variation in their length distributions. Nevertheless, some of these strains show marked biological differences, manifested in their host-parasite relations and especially in host-restriction (see p. 131). Such strains represent typical biological races of an infraspecific status, for which there is at present no provision in zoological nomenclature. In bacteriology such races are known as 'types', but the connotation of this word in biology is so wide that it is undesirable to employ it for systematic purposes. A more suitable term is 'deme' (Gilmour & Gregor, 1939; Gilmour & Heslop-Harrison, 1954), which denotes a population within a specified taxon (such as species), and may be combined with an appropriate prefix to indicate the nature of the differential criterion. Thus, in *T. evansi* strains adapted to distinct hosts represent populations differing in habitat, or ecodemes, but if it is desirable to limit the ecological criterion by specifying the hosts in question, the cameline, equine and bovine strains could be called xenodemes (cf. Hoare, 1955). A special definition should be given to the equine strains, in which, as stated above (p. 153), the mean lengths of the trypanosomes gradually increase from West to East over the geographical range of horses. According to the deme terminology, these strains represent clinodemes. It should be noted that this terminology does not claim a place in orthodox taxonomy, but applies to minor infraspecific units of variation.

(*c*) *Phylogeny*

The similarity of *T. evansi* to trypanosomes of the *Brucei* group has given rise to speculations regarding the phylogenetic implication of this relationship. Since *T. rhodesiense* and *T. gambiense* are human parasites, it was naturally assumed that *T. evansi* is more closely related to the animal parasite, *T. brucei*, which was therefore the only species considered in this connexion. To this argument can now be added the fact (established in the present study) that posteronuclear trypanosomes, when present in *T. evansi* (strain *Cc*), may be as abundant in this species

as they are in *T. brucei*, in contrast to their scantiness in typical *T. gambiense*. Apparently the first to raise the question of the ancestry of *T. evansi* was Leese (1911), who suggested that the trypanosome of Indian Surra was of African origin, 'and it does not seem unlikely that it may have been one of the Nagana group' (i.e. *T. brucei*). This thesis was developed by him further in a later publication (Leese, 1927). Somewhat similar views were also expressed by other authors (Duke, 1923; Reichenow, 1928; Lavier, 1928; Hornby, 1930), whereas Hoare & Coutelen (1933) and especially Lavier (1933, 1942–3) believed that, on the contrary, *T. brucei* might have originated from *T. evansi*.

However, my discovery of the sporadic occurrence in *T. evansi* of a degree of polymorphism comparable to that in *T. brucei*, as well as facts pointing to contacts between the areas of distribution of Surra and Nagana in Africa, have caused me to change the opinion held formerly (Hoare & Coutelen, 1933), and I am now convinced that the weight of evidence is in favour of the descent of *T. evansi* from *T. brucei*. The data supporting this hypothesis were already discussed in some previous papers (Hoare, 1940*a*, 1948*a*, *b*), and as I propose to deal with the evolution of *T. evansi* in greater detail in a future publication, I shall restrict myself here to a brief account of the conclusions arrived at in the light of the present findings.

The close morphological resemblance and affinity of *T. evansi* and *T. brucei* have now been established beyond all doubt. However, it might be argued that the monomorphism of typical *T. evansi* provides an important feature distinguishing it from *T. brucei* if it were not for the fact that the last-named species, when maintained exclusively by syringe passages through animals, loses its polymorphism and becomes monomorphic like *T. evansi*, which is also transmitted mechanically (by Tabanid flies) in nature. Furthermore, it is known that monomorphic strains of *T. brucei* lose their power to develop in tsetse-flies, and it has been demonstrated by myself and by some other workers (Hoare, 1940*a*) that *T. evansi* is also incapable of establishing an infection in these insects. It is thus seen that the appearance and the behaviour of monomorphic strains of *T. evansi* and *T. brucei* are identical. All these facts are in accord with the hypothesis that *T. evansi* had originated from *T. brucei*. This evolution could have taken place if animals infected with *T. brucei* had been introduced from an enzootic area to a new locality, free of tsetse-flies, where this trypanosome was subsequently transmitted mechanically by local Tabanids. Loss of contact with *Glossina* would in the course of time bring about the transformation of *T. brucei* into a monomorphic race indistinguishable from the species now known as *T. evansi*, in which there might occasionally be recurrences of polymorphism, representing an atavistic trait.

If this hypothesis is correct, *T. evansi* must have originated in tropical Africa, the home of *Glossina*. Evidently this evolution could only have occurred if animals outside the tsetse-zone came in contact with flies infected with *T. brucei*. In fact the clue both to such contact and to the spread of Surra outside Africa is provided by camels, which are the chief hosts of *T. evansi* in Africa throughout the vast region lying roughly north of 15–16° N. lat., which is also the northern boundary of the area of distribution of *Glossina*. The southern boundary of camel Surra and the northern boundary of tsetse-borne trypanosomiasis (including *T. brucei*)

therefore not only almost coincide, but there is also evidence that they overlap at a number of points along the dividing line (Hoare, 1940*a*, 1948*a*; Lewis, 1949; Mornet, 1954). In connexion with this hypothesis, it may be significant that the highest degree of polymorphism was exhibited in the present study by a cameline strain (*Cc*) of *T. evansi* isolated from the Anglo-Egyptian Sudan. It is conceivable that this marked polymorphism is due to the comparatively recent separation of this strain from *T. brucei* in an area where opportunities of contact with tsetse-flies cannot be excluded. In general, it would seem that infection with *T. brucei* could be contracted by camels temporarily brought into tsetse-belts, whence they spread the disease among other camels on their return to the tsetse-free areas. Having in the course of time adapted itself to mechanical transmission and lost its polymorphism, *T. brucei* acquired all the characteristics of *T. evansi*. Since the camel has for centuries been the chief transport animal in North Africa, Arabia and western Asia, the infection originally acquired in tropical Africa was probably gradually disseminated by caravans to camels in northern Africa, and eventually extended to Asia, while in some localities—especially where camels were absent—the trypanosome also adapted itself to other domesticated animals, such as equines and bovines.

VI. SUMMARY

The present revision of the morphology, taxonomy and affinities of trypanosomes causing Surra in the Old World was undertaken in order to determine the status of the diverse geographical and hostal strains, the exact relation of which to *Trypanosoma evansi* and allied species was hitherto a subject of controversy. The material for this study was represented by thirty strains from diverse mammalian hosts and various countries, extending from Morocco to the Philippines.

One of the problems dealt with is the occurrence of polymorphism in *T. evansi*. When single samples of typical (monomorphic) strains from naturally infected animals were examined, only negligible numbers of stumpy forms (0·05–0·40%) were found in a minority of cases. But in strains kept under continuous observation for several years the proportion of stumpy forms occasionally rose higher (2–25%), while one strain exhibited a remarkable and persistent degree of polymorphism in the course of 17 years, the proportion of stumpy forms—including numerous posteronuclear (= posterior-nuclear) ones—fluctuating from 0 to 60%. It was shown that—as in the case of the *Brucei* group—the length distributions in polymorphic samples of *T. evansi* could be statistically resolved into distinct curves, corresponding to the three morphological components (stumpy, intermediate, slender).

A description is given of the structure of the different forms of trypanosomes occurring in *T. evansi*, which proved to be indistinguishable from similar forms in the *Brucei* group.

A biometrical study was made of twenty-two typical (monomorphic) populations of *T. evansi*, in order to ascertain whether strains of different provenance varied in their dimensions (total length), and, if so, to determine the statistical and taxonomic significance of this variation. It was found that (*a*) the mean length of

diverse hostal and geographical strains, and even of substrains of the same strain, varied considerably, and (*b*) the difference between various samples was statistically significant in about 50 % of the pairs compared. However, from an analysis of these data it is concluded that the statistical significance of the variation in these samples does not provide criteria for distinguishing the strains taxonomically. All these strains therefore belong to one species, *T. evansi*.

The only regularity in the variation was observed in the equine strains, in which the means gradually increased in length from West to East, over the geographical range of the host. These strains correspond to clines or clinodemes.

It was also demonstrated that, when the measurements of all the populations are pooled and plotted, the variation between them is smoothed out, and the true mean characteristic of *T. evansi* as a whole ($M = 24\mu$) is obtained.

The systematic position of *T. evansi* is then considered in the light of the present investigation. A comparison of this species with members of the *Brucei* group points to a close affinity between them, on account of which *T. evansi* is classified within the *Brucei* group, the composition and diagnostic features of which have been revised and brought up to date.

The nomenclature and synonymy of *T. evansi* are discussed, and the status of the diverse infraspecific biological strains is defined in accordance with the deme terminology.

Finally, the phylogeny of *T. evansi* is considered, and a hypothesis supporting the origin of this species from *T. brucei* is advanced.

In an Appendix (by J. W. Trevan) a statistical analysis is given of the measurements of *T. evansi*.

This investigation was greatly facilitated by the help received from many quarters. For supplying the material, my thanks are due to the persons named in Table 1. To Mr C. E. Berry, Chief Technician of the Wellcome Laboratories of Tropical Medicine, I am indebted for the care he has exercised in the maintenance of the laboratory strains of trypanosomes and in the preparation of perfectly stained blood films. For invaluable help in the statistical treatment of the mensural data I wish to express the deepest gratitude to my colleagues, Dr J. W. Trevan, F.R.S., who has taken much trouble in the calculations and statistical analysis, and Dr J. C. Broom, who patiently discussed with me and enlightened me on many points. I also appreciate the courtesy of Mr A. T. Culwick and Dr K. C. Willett, for permission to quote and reproduce unpublished data, communicated to me in a letter.

APPENDIX

Statistical analysis of measurements of Trypanosoma evansi

By J. W. Trevan, F.R.S.

The statistical data for the measurements of length in twenty-two strains and substrains of the Surra trypanosomes (*T. evansi*) are given in the main paper in Table 5, and are represented graphically, by the Dice-Leraas method, in Fig. 4 (described on pp. 146–147). It is clear from this diagram that the mean length of the trypanosomes

varies more between samples than would be expected from the intrinsic variation of the samples themselves. In addition, the standard deviations vary from sample to sample. Thus, strains *A* and *C*, for which the means are practically identical, have standard deviations of 2·6 and 1·6 respectively. Estimates of the standard deviation in two samples of 100, from the same population, would be as widely different as this in less than 1 % of trials (estimated by variance ratio test). This difference in standard deviation is as strongly suggestive of a difference between the populations from which the samples are drawn as a difference of means, so that *A* and *C* are statistically different populations, although the means are almost identical. The measurements of *C*, *Ca*, *Cb* and *Cc*, on the one hand, and of *Na* and

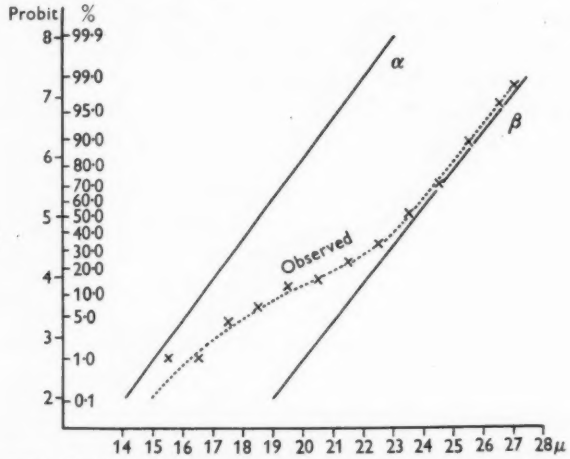

Fig. 8. *T. evansi*. Probit values for strain *A*: × × × = observed values; dotted line is found by combining 16 % of a population α and 84 % of β.

Nb, on the other, are the most crucial, in that within these two groups of samples, each of which was taken from populations of the same strain (*C* and *N* respectively), there are significant differences, either of standard deviation alone (*C* and *Cc*, *C* and *Ca*: *P* is less than 0·001; *C* and *Cb*: *P* is less than 0·01) or of means (*Na* and *Nb*, *C* against *Ca* or *Cb*, and *Cc* against *Cb*).

Some at least of these differences between mean lengths and between standard deviations are due to heterogeneity of the populations measured. To demonstrate this, the total number of trypanosomes in each count up to each recorded length were converted first to percentages of the total number counted and then to probits. The probit values were then plotted against the corresponding length plus 0·5μ (since those grouped under, say, 16μ represent individuals not greater than 16·5μ in length). If the population is a homogeneous one with a normal distribution, the points should fall on a straight line, with slight statistical variations, but if more than one normally distributed population is present in the sample measured, the line will not be straight, provided the proportions are not widely dissimilar and the means are sufficiently far apart. The method is exemplified by the plot for strain *A*

(Fig. 8). It has a pronounced inflexion, and a similar curve would be formed from a group made up of two normal populations: (α) represented by 16%, with $M = 18{\cdot}6$ and $\sigma = 1{\cdot}5$, and (β) represented by 84%, with $M = 23{\cdot}8$ and $\sigma = 1{\cdot}6$. The fitting of the components was carried out by trial. The first step is to approximate to the standard deviations of the extreme components by the slopes of lines to which the ends of the original plot are asymptotic, with the restriction that the mean of the weighted means and the sums of the weighted variances of the components must both be equal to the mean and variances of the total figures. The range of these values, giving a good approximation to the original probit plot, is limited. The solution, however, is not unique and no method is known for determining its

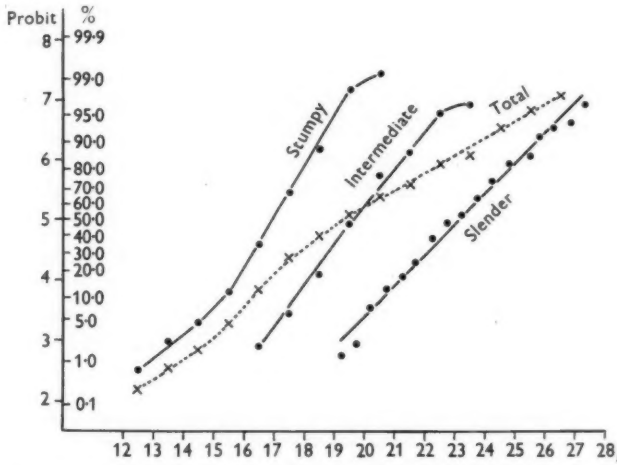

Fig. 9. *T. evansi*. Probit values for polymorphic sample of substrain *Nb* (cf. Fig. 1).

confidence limits. A better illustration is provided by the plots for the polymorphic samples of *Nb* and *Cc*, where the morphologically different 'stumpy', 'intermediate' and 'slender' forms were counted separately (see Table 3). The plots for *Nb* in Fig. 9 show that the slender forms fit a straight line fairly closely, and the intermediate forms less closely, but the falling off at the upper end is due to a few stragglers at a length of 23μ, while the stumpy forms show the presence of six below 14·5μ, which do not fit the same normal curve as the rest of the individuals. The three morphologically distinguishable forms, however, are much more nearly normal in distribution than the plot of the total observation (crosses in the figure). The plots for *Cc* in Fig. 10 show that the stumpy forms have a normal distribution but that the intermediate and slender forms are probably composite. The plot for the slender forms in particular can be fitted by two populations: (α) representing 70% with mean 26·36 and standard deviation 2·0; and (β) representing 30%, with $M = 21{\cdot}85$ and $\sigma = 1{\cdot}0$. With these parameters the calculated mean is 24·83 (observed 24·93) and the calculated overall standard deviation 2·7 is identical to that observed. The suggestion is plausible that the slender forms as counted

include a proportion of the longer members of the intermediate group. The standard deviation for group β is less than that for the intermediate group, but that is to be expected if the uncertainty of allocation was confined chiefly to the longer forms of the intermediate group. Plots of this kind have been made for all the strains studied. Few of them are straight, suggesting that there is more than one population present in each and sometimes more than the two morphological forms (slender and intermediate) characteristic of the 'typical' *T. evansi*.

This particular heterogeneity may be accounted for by the presence of slender and intermediate forms, but some of the discrepancies are so extreme that more than two normal distributions have to be assumed in order to get a reasonable fit.

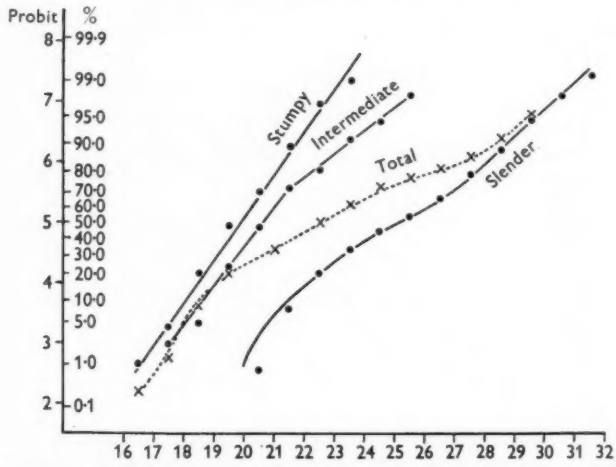

Fig. 10. *T. evansi*. Probit values for polymorphic sample of substrain *Cc* (cf. Fig. 1).

For instance, a fit for strain *J* could only be obtained by using four normal distributions. On the other hand, straight plots can be the resultant of the combination of a number of normal distributions with means fairly close together. This is best exemplified by plotting the figures for the total of all counts (=2502) for the twenty-two populations, which approaches fairly closely to a straight line of a slope corresponding to $\sigma = 3$ (Fig. 6, also cf. Fig. 5).

The occurrence of diverse populations in different proportions may account for the variations in mean length of the different counts as well as the differences in standard deviation. Strain *J*, for instance, owes its great mean length to the preponderance of one group with a mean of about 29·5 μ. A group of this average could be present in small proportion in many of the other counts. This reduces the dimensional differences among the various counts (Table 5 and Fig. 4) to a question of relative proportions of similar forms in different samples, and the problem would appear to be one not of taxonomic distinction but one of physiological factors responsible for the heterogeneous components of the population. This conclusion is buttressed by the observations on the two groups of populations, each derived from one parent strain: (*a*) *C*, *Ca*, *Cb* and *Cc*, and (*b*) *Na* and *Nb*. Both *C* and *Cb* are

substantially homogeneous as judged by the probit plot, but there is a significant difference ($P = < 0{\cdot}01$) between their standard deviations ($C = 1{\cdot}6$, $Cb = 2{\cdot}2$). It is interesting to note that the standard deviations are of the same order as that for the slender (2·0) and intermediate (1·6) forms, as graphically calculated from the straight parts of the respective plots for *Cc* (Fig. 10). This does not indicate that slender forms were entirely absent from *Cb* and intermediate ones from *C*, for if the means of the two forms were closer together in *C* than those of the slenders and intermediates of *Cc*, small dilutions of one form by the other would produce changes which might easily be obscured by casual errors of sampling. If there is anything in this resemblance between standard deviations, it must be admitted that not only is the variation in lengths between strains of different origin (hosts and localities) due to varying proportions of different normal populations, but that the means of the homogeneous elements are not stable.

The plots for the components of *Nb* (Fig. 9) are more nearly homogeneous, except for the tails of the plots. The smaller stumpy forms probably belong to a different distribution, but there seems little justification for pursuing this except to suggest that the various populations appear at different times in crops. As regards *Na*, it is also suspiciously heterogeneous.

It has thus been demonstrated that the variation in mean length of different strains of *T. evansi* is so large that this measurement is of little value in the diagnosis of this species. Moreover, there is evidence of heterogeneity in the different populations studied, which probably comprise more groups than the morphologically distinguishable slender, intermediate and stumpy forms. It is possible that this heterogeneity is due to the presence of crops of different ages.

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STRAIN VARIATION IN *ENTAMOEBA HISTOLYTICA*

II. THE EFFECT OF SERIAL LIVER PASSAGE ON THE VIRULENCE

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In previous papers (Neal, 1951*a*; Neal & Vincent, 1955) it was demonstrated that the degree of ulceration produced in rats by various strains of *Entamoeba histolytica* corresponded to the presence or absence of symptoms in the human host. In view of the well-known tendency of cases of amoebiasis to relapse clinically, it was thought that more light would be thrown on the phenomenon if the virulence of non-invasive strains of *E. histolytica* could be increased experimentally. It had been previously reported that virulence could be increased by rapid serial passage through the intestine of dogs (Faust & Swartzwelder, 1935; Meleney & Frye, 1937; Thompson, McCarthy & Reinertson, 1954) and cats (Cleveland & Sanders, 1930; Chang, 1945), but a disadvantage of this method is that the amoebae are recovered in culture with an entirely new mixture of bacteria, some derived from the animal, others from the original culture. As the liver is naturally sterile, cultures derived from experimentally produced liver abscesses will not be contaminated in this manner, the bacteria present being only those introduced into the liver with the amoebae. According to previous work, amoebic liver abscesses have been successfully produced in rats and hamsters, so it was decided to employ these animals for serial liver passage of *E. histolytica*.

MATERIAL, METHODS AND PRELIMINARY EXPERIMENTS

As the virulence of *E. histolytica* is assessed by the degree of ulceration in the caecum of rats, attempts were first made to produce abscesses in the liver of the same host. Amoebae of strains *EA* and *M*, together with their respective bacterial floras, were inoculated into the livers of rats and these were killed and examined 4 or 6 days after inoculation. One of five rats inoculated with *M* and two out of eleven with *EA* showed large abscesses at autopsy. These abscesses were thin-walled and full of thick brown pus. Small thick-walled abscesses with pus at the centre were found in six of the remaining thirteen animals, while the livers of seven were perfectly normal. Bacteria were present in all abscesses, but amoebae were recovered in culture from only one of the large abscesses. The animal with the amoebic abscess had been inoculated with *EA* amoebae and was examined after 4 days. As amoebae were found in only one rat and even then only in culture, it seems possible that the amoebae were merely surviving in a bacterial abscess. As subsequent experiments revealed, the pathology of amoebic abscesses produced in hamsters is quite different from that seen in rats.

In view of the unsuccessful results with rats, hamsters were used as laboratory hosts for liver passage, the technique of Reinertson & Thompson (1951) being used.

The amoebae were grown *in vitro* and about 25,000 amoebae, together with the concomitant bacteria, in a volume of 0·1 ml. of culture fluid, were inoculated into the right ventral lobe. The medium employed for growing the amoebae consisted of an inspissated horse-serum slope covered by egg-white diluted with Ringer's solution plus rice starch (*HSre* + *S*). The hamsters were killed and examined 5–8 days after inoculation, and those with amoebic liver lesions were examined microscopically and culturally, while those with bacterial abscesses were only cultured. The amoebae were recovered *in vitro* by inoculating *HSre* + *S* medium with a portion of the abscess. The amoebae grew readily *in vitro* without any need for treatment with acriflavine or other anti-bacterial agents, nor were any other bacteria added as the lesions were never sterile. When a further liver passage was made, the recovered amoebae were inoculated as soon as sufficient numbers were obtained, either from primary culture or after one to five subcultures.

As is described below, after a number of liver passages of strains *EA* and *M* the bacteria accompanying the amoebae increased their virulence so that the hamsters died before lesions were established. Prophylactic inoculations of bacteria were therefore given in order to raise the resistance of the hamsters before the amoebae plus bacteria were injected (Reinertson & Thompson, 1951). Bacteria were freed from amoebae by growth in nutrient broth and the absence of amoebae confirmed by subsequent growth in *HSre* + *S* medium. Hamsters were immunized by giving three intraperitoneal injections of living bacteria (grown in *HSre* + *S* medium) of the appropriate strain before inoculation with amoebae.

The virulence of the substrains after liver passage was determined by assessing the degree of ulceration (invasiveness) in the caeca of young rats. These were infected by injecting the suspension of amoebae and bacteria from culture (0·3 ml./rat) into the caecum exposed after laparotomy. The rats were killed and examined 7 days later. The presence of amoebae in the caecum was determined microscopically and culturally, and the degree of ulceration awarded a score. In maximal ulceration, the wall and contents were each awarded 4 points making a total of 8, while the total score of a normal caecum was 0 (for detailed description of rat inoculation and the scoring system see Neal, 1951*a*). In most experiments, the inocula were standardized microscopically, by placing one drop on a slide, covering with a coverglass and examining with 10 × ocular and 16 mm. objective. All inocula examined in this manner contained more than ten amoebae in each microscopic field. In a few experiments, as indicated in the tables, the numbers of amoebae were counted in a haemocytometer and the volume adjusted to contain between 250,000 and 260,000 amoebae/ml.

The strains of *E. histolytica* studied in detail were *M*, *EA* and *JO*. Strain *JO* was isolated from the faeces of a contact carrier on 2 July 1954; it was an indigenous case of amoebiasis. The histories of *M* and *EA* have been described previously (Neal, 1951*a* and Neal & Vincent, 1955, respectively). *M* was isolated from an acute case and initially proved invasive to rats, while *EA* was isolated from a chronic case and was non-invasive to rats. In connexion with chemotherapy experiments, strain *M* was used to infect a large batch of rats each week, and a close watch could therefore be kept on its virulence. It was found that the initial invasiveness

was maintained for more than 5 years (until December, 1952), but after this time its infectivity and invasiveness decreased, until at the time of writing it produces ulceration only infrequently. On the other hand, the growth *in vitro* remained constant and did not reflect the *in vivo* variation.

Other strains used were *ND*, *SI*, *HK* and *RD*. The first three strains were isolated from symptomless carriers (Neal & Vincent, 1955), while *RD* was isolated on 5 September 1950 from the faeces of a rat experimentally infected with an invasive strain (rat no. 24 in the previously published work, Neal, 1951*b*). At the time of isolation the rat had been infected for 61 weeks. Strain *RD* was initially invasive (like the parent strain *MDS*), but thereafter began to lose its invasiveness, until it is now non-invasive though infective to rats (Neal, 1954).

Substrains which have been recovered from liver lesions are distinguished by the suffix *-L* to the strain name, the Roman numeral following giving the number of passages. Where more than one substrain is isolated from different hamsters of the same passage a small letter is added in alphabetical sequence. Thus *MLVb* indicates the substrain isolated from the second hamster of the fifth liver passage of strain *M*.

It is important to note that in the following account of experiments, the terms invasive and non-invasive refer solely to the degree of ulceration caused by strains inoculated in the caecum of rats and not to the results of liver inoculation in hamsters.

RESULTS

(1) *Experiments with* M *strain*

The first liver passage in hamsters was made with *M* strain when two out of four hamsters were successfully infected. In the infected animals, the lesion had spread from the site of injection to the remainder of the lobe, and in one animal had spread across to the left lobes. The lesions were yellowish in colour and of a crumbly consistency, they were not encapsulated. In one animal, the centre of the lesion had broken down to form pus. In both animals, amoebae were observed in material taken from the liver lesion and cultures were established *in vitro*. The livers of the other two animals appeared normal except for a small scar at the site of injection. The amoebae grown *in vitro* from one of the infected hamsters were used to inoculate a further series.

The data obtained with *M* (Table 1) shows that this strain was highly virulent for the hamster liver from the commencement of the experiments. Amoebae from the first liver passage were tested for invasiveness to the rat's caecum. A total of eleven rats were inoculated intracaecally and nine became infected. Out of nine infected rats, the caeca of only three were ulcerated, giving a mean caecal score of 1·55. A portion of the liver lesion containing amoebae of the fifth hamster passage was suspended in Ringer's solution and four rats inoculated. When the rats were killed 7 days later, they were all infected with extensive caecal ulceration (mean caecal score, 7·00). Amoebae were recovered *in vitro* from three hamsters of the fifth passage, thus giving three substrains which were named *MLVa*, *b* and *c*. These substrains grew well *in vitro* like the parent strain *M*, moreover the character

of the growth, with production of a mucoid substance and gas, was also identical to that in the parent strain.

Table 1. *Proportion of hamsters developing liver lesions*

No. of animal passage	Proportion of hamsters developing amoebic lesions with different strains		
	M	*JO*	*EA*
1	2/4	1/5	2/6
2	3/6	1/4	3/6
3	2/3	1/1	5/6
4	4/4	1/5	4/5
5	3/4	1/1*	3/3
6	2/4	—	1/2
7	1/1	—	1/1
8	3/4†	—	2/2†
9	3/4†	—	3/6†
10	3/3†	—	3/3†

* Not typical amoebic abscess. Amoebae present on subculturing.
† Hamsters given prophylactic inoculations with bacteria.

The virulence of each of these substrains to the rat's caecum was compared with *M* at various intervals after recovery from the hamster's liver. The results of these experiments (Table 2) show that the increase in virulence obtained by serial liver passage is retained after cultivation *in vitro*. One substrain, *MLVa*, lost its experi-

Table 2. *The persistence of virulence to rats, acquired by three substrains after five hamster liver passages of* M *strain of* Entamoeba histolytica

Time after last passage (weeks)	Strain or substrain	Proportion of rats infected and percentage	Mean caecal score
3	*M*	2/7 (29)	0
	MLVb	7/7 (100)	4·43
	MLVc	6/7 (86)	7·00
24	*M*	10/14 (71)	2·35
	MLVa	13/13 (100)	7·30
	MLVb	10/12 (83)	4·27
	MLVc	13/14 (93)	4·71
40	*M*	11/14 (79)	3·14
	MLVa	11/13 (85)	1·91
	MLVb	13/14 (93)	3·92
	MLVc	13/14 (93)	4·77
51*	*M*	4/9 (44)	3·50
	MLVa	5/9 (55)	3·40
	MLVb	8/10 (80)	6·63
	MLVc	8/8 (100)	4·63
62*	*M*	9/14 (64)	2·00
	MLVa	7/12 (58)	2·00
	MLVb	11/15 (73)	4·27
	MLVc	11/11 (100)	5·64

* Inocula in these experiments standardized numerically.

mentally acquired virulence when tested after 40 weeks' growth *in vitro*, and subsequent tests showed that its invasiveness and infectivity were no better than those of the original strain (*M*). On the other hand, substrains *MLVb* and *MLVc* were more invasive and infective than strain *M*, and these substrains retained their acquired virulence *in vitro* for more than a year.

In view of the loss of virulence by *MLVa*, this substrain was given a further series of five hamster liver passages. After the sixth passage (the first of the second series of passages with *MLVa*), a considerable proportion of hamsters died within 2 or 3 days of injection of the amoebae. This was thought to be due to an increase in virulence of the bacteria, so in subsequent passages, hamsters were given prophylactic injections of bacteria (this problem of bacterial virulence had been encountered previously with strain *EA* and was investigated more fully, see below). This procedure reduced deaths in subsequent passages. The second series of five liver passages was commenced 63 weeks after the end of the first series.

A portion of the amoebic lesion from the last liver passage was inoculated into rats' caeca and compared with *MLVa*. The results of the rat experiment showed that there was no increase in the invasiveness (*MLVa*, score 4·50; liver abscess suspension, score 1·50), although a slight increase in infectivity was observed (*MLVa*, 4/9 infected; liver abscess suspension, 6/9 infected).

Table 3. *Results showing increase in virulence of amoebae (from substrain* MLVa*) after a second series of liver passages (substrains* MLXa *and* b)

Substrain	Proportion of rats infected and percentage	Mean caecal score
MLVa	15/26 (58)	1·80
MLVc	14/15 (93)	4·29
MLXa	24/33 (73)	2·67
MLXb	25/30 (83)	3·28

Further tests of virulence in rats were carried out with amoebae recovered *in vitro* (Table 3). These results show that the additional five liver passages increased the invasiveness and the infectivity. One substrain (*MLXa*) was little more infective than the original (*MLVa*) after liver passage, while the other (*MLXb*) increased in infectivity as well as invasiveness. The invasiveness of *MLXa* was only slightly greater than *MLVa*.

(2) *Experiments with* EA *and* JO *strains*

As it proved possible to revive the virulence of strain *M*, which had become attenuated by cultivation *in vitro*, the method was repeated with a non-invasive strain, *EA*. The results of the new series of liver passages (Table 1) showed some evidence of increasing virulence for hamster's liver up to the fifth passage where the livers of all three inoculated hamsters showed amoebic lesions. After the fifth passage the experiments were complicated by the increase in virulence of the accompanying bacteria, which resulted in the death of a considerable proportion of animals. Thus at the sixth and seventh passage four and five hamsters respectively died within 2 days of inoculation. As the livers of the dead animals were macroscopi-

cally normal at autopsy it was thought that the death was due to the bacterial component of the inoculum (see also below). Before subsequent passages, therefore, the hamsters were immunized by intraperitoneal injection of the concomitant bacteria. The deaths were then greatly reduced in number. After the fifth passage with *EA*, the centre of the amoebic lesion invariably broke down with the formation of evil-smelling pus and with an ulcer at the surface of the liver. The peritoneum and diaphragm always adhered to the lesion, and it was difficult to dissect the abscess. In passages one to five, the gross pathology of the lesions formed by *EA* strain was indistinguishable from that of *M* strain, and in all passages the lesions contained large numbers of amoebae which grew readily *in vitro*.

After the first liver passage, the amoebae recovered *in vitro* were tested for invasiveness to the rat's caecum, but although eight out of nine rats were infected no ulcers were detected in the large intestine. Next, a suspension of amoebae from a liver lesion of the third passage was injected into rats' caeca, but again, although seven out of eight were infected, only one showed low-grade ulceration (mean caecal score, 0·63). A suspension of amoebae from a liver lesion of the fifth passage infected eight out of nine rats with very small ulcers in three of them (mean caecal score, 2·13). Rats were also inoculated with amoebae grown *in vitro* from the seventh passage and with a suspension of a liver lesion of the eighth passage. Again, although seven out of eight and five out of nine respectively were infected no gross ulceration was observed (mean caecal scores, 0·25 and 0·40).

On the completion of ten liver passages, amoebae from the liver lesion and those recovered *in vitro* were compared for virulence to the rat's caecum. These results (Table 4) show that ten liver passages did not alter the infectivity or invasiveness of *EA*.

Table 4. *Comparison of caecal virulence of strains* JO *and* EA *before and after liver passage*

Strain or substrain	Proportion of rats infected and percentage	Mean caecal score of infected rats
EA	19/33 (58)	0·32
EALX cultures	14/22 (64)	0·71
EALX liver lesion suspension	5/10 (50)	0·20
JO	14/18 (78)	0·50
JOLV culture	14/25 (56)	0·93

As described above, some evidence was obtained from liver passages of strain *EA* that the bacteria of this strain had increased in virulence. Attempts were made to obtain some direct information on this point. Concomitant bacteria of the last (tenth) liver passage were freed from amoebae and the virulence compared with those of the original strain (*EA*). A similar volume of bacteria was injected into the liver in the same manner as for amoebae inoculations. Out of ten hamsters inoculated with *EALX* bacteria only six survived and liver lesions were found in all six animals, whereas all those injected with *EA* bacteria (ten hamsters) survived and only four developed liver lesions. Further evidence was given by the average

weight of the hamsters, those injected with *EA* bacteria gained an average of 0·4 g., while the group given *EALX* bacteria had lost an average of 8·8 g. The liver abscesses produced were all of the bacterial type. They were not progressive but were encapsulated with a hard wall which contained a thick white or yellowish pus. Thus there was good evidence of increased bacterial virulence by liver passage.

At the commencement of experiments with *EA*, this strain had been cultivated for more than 18 months, therefore a further series of liver passages was carried out with a more recently isolated non-invasive strain, *JO*. At the start of the liver passages this strain had been growing *in vitro* for nearly 7 weeks. As can be seen from Table 1, it proved more difficult to produce amoebic liver lesions with this strain than with the previous strains. Moreover, as in the case of the later *M* and *EA* passages, a high proportion of animals died within 2 days of inoculation from bacterial infection. The lesion observed in the final (fifth) liver passage of *JO* was not entirely amoebic in appearance, as it was partly encapsulated as occurs with a purely bacterial abscess. However, amoebae were observed directly and were recovered *in vitro* from that part of the lesion which was not encapsulated. Inoculation of rats with amoebae recovered *in vitro* from the fifth liver passage and compared with the parent strain showed that no increase in infectivity or invasiveness had occurred (see Table 3).

(3) *Experiments with other strains*

Another strain, *RD*, had decreased in virulence during prolonged *in vitro* cultivation and attempts were made to increase its virulence by liver passage. Unfortunately, only one hamster developed an amoebic lesion out of thirty-one inoculated, and the amoebae recovered from this animal did not grow well *in vitro*. As many of the hamsters developed bacterial abscesses, experiments were conducted with amoebae which had been washed in order to remove the majority of bacteria, but these experiments were also unsuccessful.

Ten hamsters were inoculated with amoebae from strain *HK*, two with *SI* and one with *ND* (all non-invasive strains), but no amoebic liver lesions were observed.

As described above in connexion with rat experiments, amoebae were sometimes recovered in culture from the bacterial abscesses.

DISCUSSION

Liver lesions in hamsters. The results of these experiments show that hamsters are more suitable for the production of amoebic liver lesions than rats. Liver lesions caused on one hand by amoebae and on the other by bacteria alone are easily distinguished macroscopically, and the former animals are more susceptible than rats. Although a liver abscess containing small numbers of amoebae was observed in one rat, it was not clear whether or not they were merely surviving in a bacterial lesion. The experiments with hamsters show that not all strains of *E. histolytica* readily induce amoebic lesions in the liver (as previously mentioned by DeLamater, Michaelson, Hallman & Blumenthal, 1954), and that the virulence for the liver does not parallel the capacity to invade the caecal mucosa in experi-

mental infections. Thus *M* was invasive in the intestine and produced liver lesions, but *EA* was not invasive in the intestine but virulent for the liver. In the case of strains *M* and *EA* there is some evidence showing that the virulence for the liver may be increased by serial liver passage, but this treatment had no effect on strain *JO*. Cleveland & Sanders (1930) also observed an increase in virulence to the liver using young cats. The gross and microscopic pathology of the liver lesions reported above was similar to that described by Reinertson & Thompson (1951). The lesions were never free from bacteria.

Although *EA* easily produced amoebic liver lesions, other non-invasive strains *JO*, *HK* and *RD* produced very few or none at all. The few animals inoculated with strains *ND* and *SI* also did not show amoebic lesions in the liver. Now there is some doubt whether the case from which strain *EA* was isolated was actually symptomless, as the clinical picture was confused by the possible presence of the sprue syndrome. From previous experiments in rats it had been argued that the symptoms in the human host were not due to *E. histolytica* (Neal & Vincent, 1955), but the present observation that *EA* easily infected hamsters' liver, whereas other non-invasive strains from undoubted symptomless carriers did not, again raises the question of the possession of some degree of virulence by *EA*. Omitting the doubtful case of *EA* strain, all other non-invasive strains isolated directly from human cases did not establish themselves in the liver of hamsters, or only with difficulty, whereas strain *M* easily infected the liver. The variation of virulence for the liver between strains may be due to the bacterial flora preventing the amoebae establishing themselves in the liver or may reflect a difference between strains of amoebae in virulence for tissue. There is some support for the latter idea from previous work. Cleveland & Sanders (1930) found that strains from dysenteric cases produced a greater percentage of abscesses in cats than those from carriers, and Yoshimura (1952) was unable to produce amoebic liver abscesses in rats with Japanese strains of *E. histolytica* but was successful with strains derived elsewhere. This he attributed to a difference in virulence. In any future research on the correlation of symptoms in the human host with virulence in laboratory animals, it would be interesting to determine the ease of production of amoebic hepatitis in addition to caecal ulceration.

Increase in virulence by animal passage. Increase in virulence by animal passage of attenuated strains of *E. histolytica* has been reported by previous workers (Cleveland & Sanders, 1930; Faust & Swartzwelder, 1955; Chang, 1945; Thompson *et al.* 1945), but they do not refer to the stability of the acquired virulence on subsequent *in vitro* cultivation. In the present work all three substrains isolated from the fifth liver passage of *M* strain retained their virulence for 24 weeks, but when tested at 40 weeks, one substrain, *MLVa*, had lost its acquired virulence. The other two substrains still retained their increased virulence after 62 weeks cultivation *in vitro*. Cleveland & Sanders (1930) inoculated amoebae into the intestine of eleven cats after four and five passages in cat's liver, but only one animal (a kitten) became infected. It was not possible to draw any conclusion regarding an increase in virulence after liver passage from these experiments.

The virulence of amoebae derived from *M* was raised a second time by a further

series of liver passages of attenuated substrain, *MLVa*, but the virulence was less than that previously obtained after five liver passages of strain *M* which, in turn, was below that originally possessed by *M*. Although the *MLX* substrains were tested in rats within 4 weeks of their isolation *in vitro*, one substrain proved less virulent (*MLXa*) than the other (*MLXb*).

Meleney & Frye (1937) found that the invasiveness of virulent and avirulent strains to kittens could be temporarily increased by serial passage in the intestine of dogs, though the acquired virulence of the virulent strains was always higher than that of the avirulent strains. The virulence returned to normal when tested after 58 weeks' cultivation in the case of the virulent strain and 19 weeks for the avirulent strain. If allowance is made for the hypersensitivity of the kitten to *E. histolytica* (cf. Neal & Vincent, 1955), these results are in agreement with the present observations using rats.

Generally speaking therefore, a factor is involved in the invasion of the large intestine by *E. histolytica*, which may be lost on prolonged *in vitro* cultivation and may be almost completely restored by liver passage in hamsters or by intestinal passage in cats and dogs. This factor is possessed naturally by strains isolated from acute cases of amoebiasis or if lost by such strains, can be restored artificially. On the other hand, non-invasive strains isolated from symptomless carriers do not possess this factor or only in a very small degree which cannot be increased experimentally.

Influence of bacterial flora. The influence of the concomitant bacterial flora on the invasiveness cannot be ignored. In the present liver passage experiments, the method prevents the introduction of additional bacteria, in contrast to intestinal passage where such modifications of the flora cannot be avoided. Although it is impossible when dealing with strains of *E. histolytica* growing with mixed and mostly unknown floras to prove the absence of contamination during liver passage, the character of the growth *in vitro* showed that gross contamination had not occurred.

In connexion with the part played by bacteria, the observation of the increase of systemic virulence by *EA* bacteria to hamsters is interesting. The data given above show quite clearly that bacteria associated with *EA* strain increased in virulence after the fifth liver passage. Yet when the amoebae and bacteria were inoculated into rats' caeca, no ulceration was observed even after ten liver passages. No evidence of increase in virulence of the *M* strain bacteria during the first series of liver passages was seen, but the invasiveness was raised. These results show that the underlying principle of increasing the virulence of strains of *E. histolytica* is not related to the increase of bacterial virulence.

Experiments have also been carried out in which bacteria from invasive *MLV* substrains have been added to the non-invasive *M*. These and other experiments bearing on the part played by the bacterial floras will be described in a later paper.

SUMMARY

1. Liver lesions were easily induced in hamsters with two strains of *E. histolytica* but not with six others.

2. Serial liver passage increased the invasiveness to the rat's caecum of one strain which had become attenuated by *in vitro* cultivation.

3. The invasiveness of one of the liver passage substrains was lost a second time after cultivation *in vitro* and a second series of liver passages restored some degree of virulence.

4. Serial liver passage did not increase the invasiveness of two avirulent strains.

5. The ease of production of amoebic liver lesions in hamsters is not correlated with invasiveness in the rat's caecum.

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STRAIN VARIATION IN *ENTAMOEBA HISTOLYTICA*

III. THE INFLUENCE OF THE BACTERIAL FLORA ON VIRULENCE TO RATS

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In previous papers it has been shown that, although all strains of *Entamoeba histolytica* are infective to rats, some produce ulceration in the caeca, whilst others do so only rarely and live as commensals in the caecal lumen. The factors responsible for this variation reside in the culture and not in the experimental host (Neal, 1951; Neal & Vincent, 1955). As the amoebae used in these investigations have always been grown *in vitro* with a mixture of aerobic and anaerobic bacteria, it is obvious that the flora may be the source of the variation of virulence. Experiments described in the present paper were designed to elucidate the influence the bacteria may exert on virulence.

This subject has already been examined by previous workers, and their results are discussed below (p. 187).

MATERIAL AND METHODS

The strains of *E. histolytica* used were as follows: strain *M* (Neal, 1951) was used early in the investigation while it was invasive and also at a later date after it became non-invasive. *MLVb* and *c* were virulent substrains derived from the attenuated *M* after the invasiveness had been restored by hamster liver passage (Neal & Vincent, 1956). Strain *RD* was also initially invasive; it too became attenuated *in vitro* (Neal, 1954), and when used for the present experiments it was non-invasive. Strain *STC* was always non-invasive (Neal, 1951) and substrain *SK*, also non-invasive, was obtained by adding *K* flora, in order to produce large numbers of cysts. All these strains were grown *in vitro* with mixed bacterial floras of aerobic and anaerobic organisms. Other strains used had known flora consisting of one (monoxenic strain*) or two (dixenic strain*) aerobic bacteria, viz $P+A^1$ (associated bacteria; *Escherichia coli*, strain A^1) and $D+pR^4$ (*E. coli*, R^4 and *Serratia marcescens*). Strain $P+A^1$ was given to me by the late Dr Dobell, and $D+pR^4$ strain was first isolated with *S. marcescens* (Neal, 1952), but as growth of the amoeba was poor, R^4—a strain of *Escherichia coli* isolated from the flora of *RD*—was added, giving strain $D+pR^4$. The growth of amoebae *in vitro* was then satisfactory.

Bacterial floras were isolated from other strains of *Entamoeba histolytica*, viz. *HN* (Neal, 1951) and *OW* (Neal & Vincent, 1955), both of which were invasive strains, and *EA* (Neal & Vincent, 1955), a non-invasive strain. For convenience, the strains are briefly listed in Table 1 according to their virulence.

* Monoxenic = amoebae grown with one associate; dixenic = amoebae grown with two associates. Nomenclature of Dougherty (1953).

The media employed for cultivation of *E. histolytica* were similar to those used previously: inspissated horse-serum slope covered with egg-white diluted with Ringer's solution (*HSre*) and whole egg slope covered with horse-serum diluted with Ringer's solution (*Ehs*). These culture media were usually supplemented with a loopful of rice starch (+*S*). The usual bacteriological media were used for isolation of various bacteria.

Table 1. *Characteristics of strains of* Entamoeba histolytica *used, with their virulence to rats*

Invasive: *M*, *MLVb*, *MLVc*, *HN*, *OW*

Non-invasive: *M**, *RD**, *STC*, *SK*, *P*+*A*[1]†, *D*+*pR*[4]†, *EA*

* Attenuated strain.
† Strains with known bacterial associates.

The bacteria accompanying the amoebae were usually separated from the amoebae in their original mixed state without differentiation of aerobes and anaerobes, by inoculating nutrient broth with a portion of the sediment from a culture of the desired strain of *E. histolytica* (Dobell, 1952). The broth was then incubated for 24 hr. at 37° C. which generally killed the amoebae, although a second subculture in broth sometimes proved necessary. In all cases, the absence of amoebae was confirmed by inoculating the bacteria into *HSre*+*S* medium and examining for amoebae after 4 to 5 days' incubation. The bacterial floras were then kept at room temperature until required. Before adding to a culture of *E. histolytica*, the bacteria were grown for 24 hr. in *HSre*+*S* medium.

As it is possible that not all the bacteria would survive growth in nutrient broth, followed by storage for several months at room temperature, the bacterial floras of some strains were isolated more carefully. These floras (used in experiments described in 1*d*, 2*b* and 2*c*, see below) were freed from amoebae by subculturing twice a week in *HSre* medium without starch and keeping them continuously at 37° C. Two or three subcultures were generally sufficient to eliminate amoebae. Another method—used with strain *EA*—consisted in allowing a culture of starch-free amoebae in *Ehs* medium to remain at room temperature overnight (*ca.* 16° C.). As there was no change of medium in these two methods, there was a greater chance of obtaining the bacterial floras unaltered; further, the bacteria were grown continuously at 37° C., except for approximately 16 hr. in the case of *EA* bacteria. Absence of amoebae was always confirmed as described in connexion with the broth method.

Virulence tests of the cultures of *E. histolytica* with various combinations of bacteria were made by injecting the amoebae into the caeca of young rats. The animals were killed 7 days after inoculation, and the caecal contents examined microscopically for amoebae and, if negative, cultured in *HSre*+*S* medium. The virulence or degree of invasiveness was measured by determining the mean caecal score of infected rats. This was done by examining the caecal wall of each animal macroscopically for presence of ulceration, and awarding a score (extreme ulceration = 8, normal = 0). For further details of the animal tests, see Neal (1951).

The inoculum consisted of a 2-day growth of amoebae with bacteria in suitable culture medium (usually *HSre*+*S*). The number of amoebae per volume was not standardized numerically, but a drop examined microscopically with the 16 mm. objective and 10 × ocular showed at least ten amoebae in each field.

The method of killing all aerobic bacteria was that described by Dobell (1952), and consisted in treating cysts with 0·1% acriflavine for 10–14 days at 4° C.

RESULTS

The experiments fall into two series. The first deals with the addition *in vitro* of total bacterial floras isolated free of amoebae from strains of *E. histolytica* to other strains of this species; after incubation for a short period with the new mixture bacteria, these cultures were tested for virulence in rats. The second series deals with attempts to revive the virulence of attenuated strains by adding bacteria isolated from virulent strains.

Series 1

(*a*) The bacteria from virulent strain *HN* were added to the amoeba plus bacteria complex of the non-invasive strain *STC*. The *HN* bacteria were given 24 hr. growth in *HSre*+*S* medium, then the amoeba and bacteria of strain *STC* were added. Amoebae of strain *STC* were subcultured eight times in this manner. The virulence of this 'additive' strain was then compared with the original strain. The results (see Table 2) showed no significant increase in virulence.

(*b*) Next, the flora of *STC* was partially purified by killing aerobic bacteria with acriflavine. Strain *STC* itself did not produce sufficient numbers of cysts for treatment with acriflavine, so it was first necessary to add *K* flora to increase the encystation of *STC* amoebae. Cysts of the new strain *SK* were treated with acriflavine, then hatched with a pure strain of *Escherichia coli* (A^1), and the total floras from the virulent strains of *Entamoeba histolytica M* and *HN*. After several subcultures had shown that the amoebae were growing satisfactorily with the new bacterial floras, the virulence of the 'synthetic' strains was compared with the parent strain (*SK*) and *M*. The results in Table 2 show that neither the addition of *K* bacteria nor the bacteria from the invasive strains *M* and *HN* had any significant effect upon the invasiveness of *STC*.

(*c*) The concomitant bacteria from strains of *E. histolytica M* and *OW* were then added to strains with known floras of one or two aerobic bacteria. The bacteria from strain *M* were added to the monoxenic strain $P+A^1$ and to the dixenic strain $D+pR^4$. As bacteria from *HN* strain showed no effect on *STC* amoebae in previous experiments, the bacteria from another invasive strain of *E. histolytica*, *OW*, were added to $D+pR^4$, as well as those from strain *M*.

Inoculation of rats with these 'synthetic' strains showed no detectable increase in virulence. In fact, strain $P+A^1$ would not infect rats at all and the addition of the total flora from *M* did not make it infective.

(*d*) The final experiments in this series were designed to determine whether the avirulence of a strain was due to an inhibiting action on the amoebae by the accompanying bacteria. Amoebae from the invasive strain of *MLVc* were washed

Table 2. *Summary of virulence tests in the rats of* Entamoeba histolytica *in association with various bacteria*

Group of experiments	Strain of *E. histolytica*	Strain of *E. histolytica* from which bacteria were derived	Results of rat inoculation: Proportion of rats infected (percentage in brackets)	Results of rat inoculation: Mean caecal score of infected rats
Series 1				
(*a*)	*STC*	—	10/16 (63)	0·40
	STC	*HN*	15/16 (94)	1·25
(*b*)	*SK*	—	12/16 (75)	1·33
	SK	*A*[1]*	10/13 (77)	0·20
	SK	*M*	20/23 (87)	1·00
	SK	*HN*	31/35 (89)	1·90
	M	—	11/12 (92)	7·45
(*c*)	*D*+*pR*[4]	—	17/30 (57)	1·13
	P+*A*[1]	—	0/4 (0)	0
	D+*pR*[4]	*M*	5/9 (56)	0
	D+*pR*[4]	*OW*	0/3 (0)	0
	P+*A*[1]	*M*	0/4 (0)	0
(*d*)	*MLVc*	—	11/12 (92)	5·64
	MLVc	*MLVb*	9/10 (90)	4·33
	MLVc	*EA*	7/9 (78)	5·71
Series 2				
(*a*)	*M*†	—	22/37 (59)	0·68
	M†	*M*	6/10 (60)	2·60
	M†	5*M*	16/28 (57)	0·81
	RD†	—	20/22 (91)	0·75
	RD†	*M*	14/18 (78)	0·86
(*b*)	*M*†	*M*†	4/9 (44)	0
	M†	*MLVc*	3/8 (38)	0
(*c*)	*M*†	—	12/22 (55)	2·17
	MLVb	—	5/12 (42)	3·10
	MLVc	—	13/14 (93)	5·69
	M†	*MLVb*	5/12 (42)	2·80
	M†	*MLVc*	5/10 (50)	1·00

* Pure strain of *Escherichia coli*.
† Attenuated strains.

three times with Ringer's solution to reduce the number of bacteria, then inoculated into one tube of *HSre*+*S* medium containing bacteria from a non-invasive strain (*EA*) and another with bacteria from an invasive strain (*MLVb*, parallel strain of *MLVc*). The virulence of these 'additive' strains (see Table 2) was similar: they were as invasive as the original *MLVc*.

Series 2

(*a*) After the above experiments with *M* strain had been performed, it became attenuated by prolonged *in vitro* cultivation (compare results obtained with *M* strain in series 1*a*, with series 2). As the bacterial flora, isolated while *M* strain

was still virulent, was available, attempts were made to revive the virulence with its own flora. Amoebae from the attenuated *M* strain were inoculated into *HSre*+*S* in which the *M* flora had been given 24 hr. incubation. As the virulence of the 'additive' strain showed some increase (Table 2), further attempts were made to swamp the bacteria of the attenuated strain by repeatedly subculturing the amoebae in *HSre*+*S* seeded with *M* bacteria from the initial invasive phase. This was carried out five times (5*M*). Results of virulence tests, however, showed no confirmation of increased virulence. Moreover, the first experiment of this section was repeated without any increase of virulence being observed.

This type of experiment was repeated with another attenuated strain of *E. histolytica*, *RD* and the *M* bacteria, with similar negative results.

(*b*) Previous work had shown that the invasiveness of the attenuated *M* strain could be increased by serial liver passage (Neal & Vincent, 1956). Therefore, bacteria from substrain *MLVc* (whose virulence was restored by liver passage) were added to washed amoebae of attenuated *M*, but no increase in invasiveness was found.

(*c*) Although in the above experiments certain bacterial floras were added to other strains, there is no way of telling, except with the monoxenic strains, if all the added bacteria survived. As a last attempt to raise the invasiveness by means of bacteria, rats were inoculated intracaecally with amoebae immediately followed by additional bacteria. Thus a group of rats were inoculated with attenuated *M* amoebae, followed, in one group, by bacteria from the invasive strain *MLVb* and, in another, by bacteria from *MLVc*. In this way it was certain that the additional bacteria reached the rats' caecum. Again, however, no increase of virulence was observed (see Table 2).

DISCUSSION

These experiments show that the addition of bacteria from invasive strains of *E. histolytica* to non-invasive strains will not increase the virulence of the non-invasive strains, nor would bacteria from non-invasive strains decrease the virulence of invasive strains. It was also found that, although the virulence of strain *M* was increased by serial liver passage (Neal & Vincent, 1956), the bacteria from the invasive passaged strains would not revive the virulence of the attenuated *M*.

No attempt was made to analyse the bacteria from these strains of *E. histolytica*. If the addition of bacteria had resulted in an increase in virulence, the next step would have been to separate the components of that mixture but, in the absence of any increase of virulence, it was not done.

It seems obvious that monoxenic and dixenic strains are of limited value, since the biological properties of the amoebae seem to be altered after prolonged growth *in vitro*. Thus, in the present work $P+A^1$ proved non-infective to rats, although previously this strain had an infectivity of 75%; similarly, $D+pR^4$ lost its infectivity to rats. Luttermoser & Phillips (1952) also found that the infectivity of *E. histolytica* dropped in monoxenic culture with organism *t*.

In previous work on the influence of bacteria upon the intestinal virulence of strains of *E. histolytica*, kittens have been mainly used as experimental hosts.

It is well known that these animals are very susceptible to infection with *E. histolytica* and die in about a fortnight from bacteraemia. Therefore, if virulent bacteria were administered in addition to the amoebae, it would be expected that the animal would die sooner, and with greater ulceration. This effect has, in fact, been demonstrated. Spector (1935) found that the virulence was increased by a haemolytic *Streptococcus*, 'green producing streptococcus', pneumococcus types I and II, *Pseudomonas pyocyaneus* and *Escherichia coli*; Deschiens (1938) used *Salmonella typhi*, *S. paratyphi* and *Escherichia coli*; and Nauss and Rappaport (1940) used a green fluorescent bacillus allied to *Pseudomonas chlororaphis*. Experiments with rats (Stewart & Jones, 1948) gave slightly different results. Although the infection in the rodent is less severe than in the kitten, the addition of virulent *Escherichia coli* and *paracolon* bacteria, isolated from cases of dysentery, aggravated the lesions but did not affect the mean degree of ulceration.

Meleney & Frye found that strains of *Entamoeba histolytica*, when examined carefully in kittens, showed constant differences in virulence (Meleney & Frye, 1933). These workers then proceeded to interchange the floras of two strains and retest them. The results (Frye & Meleney, 1933) showed that the new floras did not affect the virulence. These workers also added virulent haemolytic streptococci isolated from a human blood stream infection to an avirulent strain of *E. histolytica*, but observed no rise in virulence. They concluded that the associated bacteria played no part in the virulence of these strains of *E. histolytica*. These results differ from those of other workers, in that the bacteria occurring naturally and cultured together with the amoebae, were interchanged, whereas other workers used bacteria often known to possess virulent properties and which usually were isolated from diseases other than amoebiasis. In reviewing the work of previous authors, greatest importance should be attached to those experiments using strains of *E. histolytica*, known to show differences of virulence in laboratory animals, since these probably afford the best method of elucidating the part played by the bacteria.

Recently, Okamoto (1954) has published some results which showed that *Clostridum perfringens* did not influence the invasiveness of the amoebae. However, Okamoto used large rats (weighing 45–71 g.) and, as mentioned previously (Neal & Vincent, 1955), this reduces the value of his results because older rats are more resistant to infection and ulceration.

It seems, therefore, that the previous and present experimental work can be best explained by assuming that two different phenomena are involved. First, virulent bacteria, if present, will aggravate existing amoebic lesions in susceptible hosts; secondly virulent bacteria or floras from virulent strains of *Entamoeba histolytica* will not induce amoebae of avirulent strains to produce ulceration as severe as that caused by virulent strains of *E. histolytica*. The first tenet is supported by clinical evidence, as it has been shown that treatment of chronic amoebic dysentery with antibacterial drugs will facilitate eradication of the amoebae by subsequent treatment with standard amoebicides (Hargreaves, 1946). The second observation suggests that the ulceration produced in kittens, rats and possibly man by *E. histolytica* depends primarily upon the invasive capacity of the amoebae.

Other evidence in support of the second part of this theory is derived from liver passage experiments (Neal & Vincent, 1956). This work showed that although the virulence of bacteria accompanying non-invasive amoebae was increased by five serial passages, the amoebae remained non-invasive after as many as ten liver passages. Conversely, the virulence of bacteria of an attenuated strain of *E. histolytica* did not increase after five liver passages, while the virulence of the amoebae was restored. After ten liver passages, however, the bacterial virulence was raised, though there was not a corresponding further increase in invasiveness.

The conclusions which have been reached concerning the primary responsibility of the amoeba in producing intestinal lesions are equally applicable to liver-invasion. DeLamater, Michaelson, Hallman & Blumenthal (1954) found that some strains of *E. histolytica* would not produce liver lesions in hamsters, in contrast to the high liver infectivity of other strains. They have also shown that reversal of the bacterial floras of infective and non-infective strains did not alter their virulence. Similar results were also observed by Reinertson (in a personal communication to DeLamater *et al.* 1954).

Cleveland & Sanders (1930) observed that *E. histolytica* grown with *Bacillus brevis* (a non-pathogenic, spore-forming aerobe) would not produce liver abscesses in cats, whereas, if bacteria from a virulent strain were added, abscesses were formed. These results are apparently contradictory to the above observations. From the available evidence, a possible explanation is that the amoebic growth was not normal with *B. brevis*.

Some interesting work has been carried out by Phillips and his colleagues on the role of bacteria in the virulence of *Entamoeba histolytica*. They first used rabbits as experimental hosts and found that monoxenic cultivation of *E. histolytica* with a bacterium called organism *t* resulted in a partial loss of virulence, but monoxenic cultivation with *Trypanosoma cruzi* caused a complete loss of infectivity (Luttermoser & Phillips, 1952). Later work (Phillips and Bartgis, 1954) using guinea-pigs, showed that the *Entamoeba histolytica–Trypanosoma cruzi* cultures were still virulent if tested not later than 8 weeks from the time of isolation. This suggests that the amoeba itself was invasive. Recently, further work has been carried out using guinea-pigs, which had been reared in a sterile manner, then infected with a single bacterial species and subsequently inoculated with amoebae from an *Entamoeba histolytica–Trypanosoma cruzi* culture. The results showed that the amoebae did not survive in bacteria-free animals but successfully infected, with ulceration of the intestinal wall, guinea-pigs harbouring *Escherichia coli* or *Klebsiella aerogenes* (= *Aerobacter aerogenes*) (Phillips, Wolfe, Rees, Gordon, Wright & Reyniers, 1955). These experiments therefore show that certain bacteria are necessary to permit the full development of the invasive properties of *Entamoeba histolytica*. It would be interesting to repeat these experiments using a non-invasive strain.

SUMMARY

1. The total bacterial floras from invasive strains of *Entamoeba histolytica*, when added to non-invasive strains, do not increase the invasiveness of the latter.

2. The bacterial flora of non-invasive strains do not inhibit the invasiveness of virulent strains.

3. Bacteria from a virulent substrain (revived by serial liver passage) do not increase the invasiveness of the attenuated parent strain.

4. It is concluded that the virulence of a strain of *E. histolytica* is primarily dependent upon the invasiveness of the amoeba itself.

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A STUDY OF THE FACTORS AFFECTING THE EMERGENCE OF THE GAMETOCYTES OF *PLASMODIUM GALLINACEUM* FROM THE ERYTHROCYTES AND THE EXFLAGELLATION OF THE MALE GAMETOCYTES

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I. INTRODUCTION

Since the emergence of the gametocytes of Haemosporidia from the erythrocytes and the 'exflagellation' of the male gametocytes were never observed in blood until some minutes after it had been withdrawn from the body, early workers believed that a drop in temperature must be an important factor in producing these changes. Danilewsky (1889) found that 'excapsulation', i.e. the emergence of the gametocytes from the erythrocytes, and the exflagellation of the male gametocytes did not occur in blood maintained at 40° C., though both occurred in similar preparations of blood from the same source if they were maintained at 20–25° C.; he therefore concluded that these changes were brought about by the drop in temperature. In observations upon *Haemoproteus* in the blood of falcons Claus (1903) found that exflagellation took place in blood kept at 33° C., but not in that kept at 35° C., although the gametocytes emerged from the erythrocytes at this higher temperature. Ross (1897) did not believe that a drop in temperature could be an important factor in inducing exflagellation, as the temperature at Secunderabad where he was working was nearly that of the human body; on the occasion when he

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first observed exflagellation in the stomach of the mosquito, the temperature of his laboratory was 95° F. (35° C.) (Manson, 1896). Ross (1897) found that in blood rich in crescents exflagellation was very rare if air was excluded from the blood by vaseline, an observation confirmed by Claus (1903), and that when blood was examined under cover-slips exflagellating parasites were found most frequently near to air bubbles. Ross, however, did not pursue these observations upon the effect of air, but attributed exflagellation chiefly to a change in the density of the blood.

No further studies of the factors responsible for the emergence of the gametocytes from the red blood corpuscles and the exflagellation of the male gametocytes were made until some 25 years later, when Marchoux & Chorine (1932) carried out a series of experiments upon the gametocytes of *Haemoproteus orizivorae* from the blood of *Padda orizivora*, and *Haemoproteus columbae* from pigeons. They found that the emergence of the gametocytes from the erythrocytes, exflagellation of the male gametocytes, fertilization and formation of ookinetes took place in drawn blood at temperatures of 13–43° C., with an optimum at 37° C. Since the normal body temperature of birds ranges from approximately 40 to 42° C., a drop in temperature below that of the vertebrate host could not be the factor which initiated these changes.

Since blood loses CO_2 when exposed to air, Marchoux & Chorine turned their attention to the effect of this gas upon the gametocytes. When blood containing gametocytes of *Haemoproteus* was exposed to CO_2, the gametocytes failed to emerge from the corpuscles, but they emerged and developed normally if the blood was subsequently exposed to air, provided that the exposure to the CO_2 had not been too prolonged. The presence of O_2 was not essential for the development of the gametocytes, which proceeded normally in an atmosphere of H_2.

Marchoux & Chorine found that the pH of freshly drawn chicken blood, as determined by the Sannié electrode, was 7·3–7·4, but if the blood was exposed to the air as a thin layer in a Petri dish, the pH rose to 7·75 in 5 min. When parasitized pigeon blood was added to an equal volume of Tyrode's solution, adjusted so that the pH of the mixture was 7·6, the gametocytes developed normally, but if the pH of the Tyrode's solution was such that the pH of the blood-Tyrode mixture was 6·13 the development of the gametocytes was totally inhibited. This inhibition, however, could be reversed by the addition of alkaline Tyrode's solution to give a pH of 7·6. Marchoux & Chorine concluded that, in the body of the host, the development of the gametocytes was inhibited by the presence of CO_2 in the blood, and that the action of the CO_2 was due to its effect upon the pH of the blood. Similar results were obtained by Chorine (1933) upon *Plasmodium praecox* (= *P. falciparum*).

More recently, Micks, de Caires & Franco (1948) studied the pH of the stomach content of *Culex pipiens*, *C. quinquefasciatus* and *Aëdes aegypti* which were unfed, and of individuals which had fed upon duck blood. With a few exceptions the pH of the blood from the mosquitoes' stomachs was approximately the same as, or below, that of duck or canary blood. Since *P. elongatum* exflagellated readily in the stomachs of the two species of *Culex*, Micks and his co-workers were unable to correlate exflagellation with a change in pH.

The following experiments were made in an attempt to obtain further knowledge of the factors influencing the development of the gametocytes when infected blood is withdrawn from the vertebrate host, either by syringe or by mosquitoes. In this work reference to the development of the gametocytes means only their emergence from the infected erythrocytes and exflagellation of the male gametocytes; other aspects of development such as division of the nuclei, maturation and fertilization were not studied.

II. METHODS

The strain of *Plasmodium gallinaceum* used in the following experiments was maintained by mosquito passage through chicks 5 days to 5 or 6 weeks old, the larger birds being used in experiments requiring relatively large quantities of blood. The chicks were infected either by the bite of varying numbers of *Aëdes aegypti* from batches in which the rate of infection was known to be high, or by intravenous injection of a suspension of sporozoites in inactivated chick-serum diluted 1:1 in isotonic saline (1·025 g. NaCl in 100 ml. of glass-distilled water, Heilbrunn 1952). In infections induced by large numbers of sporozoites, whether by intravenous injection or by bite of infected mosquitoes, the number of gametocytes produced was usually much greater than in a strain maintained by blood-inoculation, although parasitaemia was seldom so heavy and the period of acute blood infection was much briefer. Only blood containing large numbers of mature gametocytes was used in the experiments. Blood-inoculated infections were used in some of the estimations of the pH of infected blood since in these experiments very heavily infected blood was necessary.

Observations were made upon blood diluted with citrate-saline (15 g. sodium citrate and 4 g. sodium chloride in 1 l. of glass-distilled water, Marchoux & Chorine, 1932) or with heparin (1 mg. heparin in 1 ml. 0·85 % NaCl in glass-distilled water). The addition of heparin to the blood was found to have no injurious effect upon the gametocytes; their emergence from the erythrocytes and the exflagellation of the male gametocytes took place as rapidly and as frequently in heparinized blood as in blood from the same source mixed with citrate-saline, if the physical conditions, e.g. temperature, the volume of blood and its surface area exposed to air, were similar. In heparinized blood exposed as a thin film to moist air—the classical method for testing for exflagellation—exflagellation occurred as rapidly as in untreated blood exposed in the same way.

Measurements of the hydrogen-ion concentration of the blood were made with a Pye Universal pH meter and a micro-glass electrode designed by Hartree (1952). Since the electrode consisted of a long capillary glass tube, loss of CO_2 from the blood whilst the reading was being taken could occur only from the small area exposed to the air at the top of the electrode tube. The diameter of the capillary tube was so small, and the wall so thin, that the adjustment of the temperature of freshly drawn blood to that of the electrode would be very rapid. The pH meter was standardized against a standard buffer of pH 4 or pH 7, and the meter was checked against this standard after each series of readings.

The pH of the blood from the stomachs of mosquitoes recently fed upon chickens

was estimated by the micro-glass electrode described by Bishop, Hartree & McConnachie (1956).

All experiments were carried out at the temperature of the laboratory (18–26·5° C.). Glassware, including slides and cover-slips, was of hard glass, either Pyrex or Chance.

III. THE EFFECT OF CARBON DIOXIDE AND NITROGEN UPON THE DEVELOPMENT OF THE GAMETOCYTES OF *PLASMODIUM GALLINACEUM*

In the following experiments upon the *in vitro* effect of CO_2 and N_2 upon the gametocytes of *P. gallinaceum*, vacuum tubes (11 × 1·3 cm.), with a side arm inserted 6·5 cm. from the base, were used. The upper end of the tube was closed by a rubber bung through which passed a narrow glass tube. The gas was passed into the vacuum tube through the side arm and escaped through the tube in the bung. The gas, obtained from a cylinder, was bubbled through a column of normal saline, 30 cm. high, by means of a sintered glass gas diffuser of fine porosity, to humidify it so that evaporation should not take place when it was passed over the blood. The saline was saturated with the gas before the experiment began. In the control experiments air was bubbled through a column of saline in a similar manner by an electric pump.

One ml. of citrate-saline (p. 194) was added to each of two vacuum tubes, and the gas to be tested passed through one of them for 10 min., the tube being shaken repeatedly to ensure that the gas dissolved in the citrate-saline; air was passed through the control tube for the same length of time. Immediately after withdrawal from the jugular vein of the bird, 1 ml. of blood rich in gametocytes was added to each of the tubes, which were shaken gently from time to time in order that the gas should dissolve equally through the blood-citrate-saline mixture. Samples of blood were removed from each of the tubes, at 5 min. intervals, by capillary pipettes, a clean pipette being used for each sample. Unless stated otherwise, the sample was covered with a cover-slip and immediately examined, for 2 min., under the microscope.

In the first experiment CO_2 was passed through one tube and air through the other. Exflagellating microgametocytes were numerous in the sample taken from the control tube 20 min. after the beginning of the experiment, but none was observed in the sample from the blood exposed to CO_2. After 30 min. the stream of CO_2 was replaced by air, but although samples of the blood were examined for a further period of 1 hr. 20 min., and the tube shaken frequently, no exflagellation was observed. In a second similar experiment blood rich in gametocytes was exposed to CO_2 for 30 min. No exflagellation was seen in samples of blood removed during this period, covered immediately with a cover-slip, and examined without delay. Other samples were exposed to the air for 3 min., as drops on slides, before being covered with a cover-slip. In several of these samples, removed either whilst the blood was exposed to CO_2 or after the gas had been replaced by air, exflagellating microgametocytes were seen 21–28 min. after the sample was removed from the tube; but in some samples, removed either whilst the blood was exposed to CO_2

or later, no exflagellation occurred after exposure to air. In samples of control blood exflagellating microgametocytes were first observed 20 min. from the beginning of the experiment.

In these experiments, in which the gametocytes were exposed to an atmosphere wholly of CO_2, the conditions were very different from those *in vivo*; in an attempt to make them more comparable a mixture of 5 % CO_2 + 95 % O_2 was used. In the first experiment blood rich in gametocytes was exposed to this gas mixture for 30 min. No exflagellation was seen in samples removed during this period, covered immediately, and examined for 2 min.; but when the gas mixture was replaced by air, exflagellating microgametocytes were present in a sample removed 20 min. after the replacement of the CO_2 mixture by air. In the control blood a few exflagellating gametocytes were observed 13 min. from the commencement of the experiment and they were numerous 3 min. later. In a second experiment, blood rich in gametocytes was exposed to 5 % CO_2 + 95 % O_2 for 60 min. No exflagellating parasites were found in samples of blood removed during this period, but one was found in a sample removed 15 min. after the gas mixture was replaced by air, and they were numerous in a sample removed 3 min. later. In the control, exposed to air, exflagellation was observed 15 min. after the blood was withdrawn from the bird. In a third, similar experiment, in which blood rich in gametocytes was exposed to the gas mixture for 60 min., no change in the gametocytes was observed in any sample of blood removed during this period, although exflagellation was observed in the control blood 15 min. from the beginning of the experiment; but when the gas was replaced by air, gametocytes were seen to emerge from the erythrocytes and exflagellation occurred within 15 min.

To test whether the highly oxygenated state of the blood produced by the 5 % CO_2 + 95 % O_2 had any effect upon the gametocytes, citrated blood rich in gametocytes was exposed to a mixture of 5 % CO_2 + 95 % N_2. In the two experiments performed, the results were similar to those with 5 % CO_2 + 95 % O_2, exflagellation occurring within 15 min. of the replacement of the gas by air in one experiment, and within 25 min. in the other. In the two controls exposed to air, exflagellation occurred within 15 and 20 min.

In blood exposed to an atmosphere of pure nitrogen exflagellation occurred as rapidly and frequently as in the control exposed to air. Absence of oxygen therefore had no inhibitory effect upon the development of the gametocytes.

Under the conditions of our experiments 5 % CO_2 completely inhibited the development of the gametocytes of *P. gallinaceum*; neither male nor female gametocytes emerged from the erythrocytes whilst exposed to this concentration of gas, but the gametocytes of both sexes emerged from the erythrocytes within the first few minutes of the 5 % CO_2 being replaced by air, and exflagellation of the male gametocytes occurred after a similar period to that in which it occurred in freshly drawn blood exposed to air. Whereas the gametocytes appeared to be undamaged by the exposure to 5 % CO_2, recovery from exposure to undiluted CO_2 was very uncertain.

The results of these experiments confirm those of Marchoux & Chorine upon the inhibitory effect of CO_2.

IV. COMPARISON OF THE pH OF NORMAL CHICKEN BLOOD AND BLOOD HEAVILY INFECTED WITH *PLASMODIUM GALLINACEUM*

In view of the conflicting opinions on the importance of a rise in the pH of the blood in stimulating the development of the gametocytes, the relationship of the pH of the blood to the development of the gametocytes of *P. gallinaceum* was studied *in vitro*. Measurements were made of the pH of normal and infected chicken blood, and of the changes occurring on exposure to air. In the earlier experiments, 5·5 ml. of blood were drawn from the jugular vein of a chicken into a syringe containing 0·5 ml. of heparin-saline (p. 194), care being taken to prevent the entry of air bubbles into the syringe. The heparinized blood was immediately expressed, in 1 ml. volumes, into each of five 25 ml. Erlenmeyer flasks, on the base of which it formed a thin layer. In blood exposed to air in this manner, the gametocytes emerged from the erythrocytes within 10 min. of withdrawal from the chicken, and exflagellating microgametocytes were first found within 12–20 min. It was therefore assumed that any change in the pH of the blood which affected these developments must occur soon after the blood had been withdrawn from the body. The pH of the blood in the first flask was therefore determined without delay, and that of the blood in the remaining flasks at 5 min. intervals, one measurement being made upon each flask.

In a series of measurements (Table 1, group A), made with the Hartree glass electrode upon blood from a group of normal birds, the initial readings varied from pH 7·50 to 7·63, and although a rise of pH 0·21–0·34 occurred over the 20 min. period, it was steepest in the first 15 min. No significant difference was observed between these pH measurements and those made by the same method upon infected blood.

In man, according to Van Slyke (1921), although the pH of the blood of a given individual appeared to be very constant, that of different individuals varied from 7·25 to 7·45, with a mean of 7·35. The pH of the blood of a normal fowl, measured with a Sannié electrode, was found by Marchoux & Chorine (1932) to be 7·3–7·4, whilst that of pigeons, estimated by a colorimetric method, varied from 7·16 to 7·42. It was thought that the high pH values in the initial readings in group A, as compared with those of Marchoux & Chorine, might be due to the diffusion of CO_2 from the blood before it was put into the glass electrode. To reduce the time of exposure of the blood to the air to a minimum, in a further group of experiments (Table 1, group B) blood was withdrawn from the bird and a little expelled from the syringe without delay, directly into the glass electrode. A measurement made thus is called the immediate pH. The remainder of the blood was exposed to the air, in 1 ml. volumes in 25 ml. Erlenmeyer flasks, as in the previous experiments, and the pH measurements made upon the series at 5 min. intervals. The immediate measurements made upon normal blood showed little variation and were approximately similar to the lower initial readings of the previous group; and the rise in pH during the first 5 min. exposure to air was equal to, or slightly greater than, the biggest rise recorded over the similar period in group A. Three of the immediate measurements made upon heavily infected blood were lower than those made

upon normal blood in either group, but the three higher values fell within the range of normal blood in group A.

The pH measurements in groups A and B were all made upon a mixture of heparin-saline and blood in the proportion of 1:11. The pH of the heparin-saline

Table 1. Plasmodium gallinaceum. *The* pH *of normal and infected chicken blood exposed to air*

Group	Blood	Temperature (° C.)	pH Initial	pH Time of exposure to air in minutes 5	10	15	20
A	Normal	24	7·52	7·62	7·68	7·73	7·73
	Normal	24	7·63	7·73	7·78	7·88	7·97
	Normal	25	7·60	7·68	7·80	7·90	7·92
	Normal	25	7·60	7·75	7·82	7·87	7·90
	Normal	25	7·55	7·67	7·76	7·88	—
	Normal	26	7·52	7·68	7·70	7·76	7·84
	Normal	26·5	7·54	7·70	7·76	7·80	7·78
	Normal	26·5	7·50	7·60	7·65	7·67	7·74
	Infected	18	7·63	7·78	7·90	7·94	7·88
	Infected	18	7·54	7·70*	7·74	7·80	7·85†
	Infected	20	7·69	7·84*	7·93	7·90†	7·96
	Infected	21	7·62	7·80*	7·92†	7·88	7·97
	Infected	21	7·47	7·57	7·63	7·68	7·70
	Infected	21	7·58	7·68	7·80	7·83	7·86
	Infected	23	7·58	7·78*	7·94†	7·97	7·97
	Infected	24	7·66	7·78	7·89	7·93	7·97
	Infected	25	7·61	7·73	7·85	7·90	7·90
	Infected	25	7·53	7·66	7·73	7·78	7·70
	Infected	25	7·64	7·86	7·93	8·00	7·96
	Infected	25	7·52	7·62	7·72	7·72	7·67
			Immediate				
B	Normal	22	7·53	7·68	7·78	7·83	7·93
	Normal	22	7·51	7·75	—	—	—
	Normal	22	7·51	7·72	7·81	7·87	7·86
	Infected	21	7·33	7·50	—	7·56	7·63
	Infected	21	7·40	7·55	7·61	7·66	7·69
	Infected	21	7·52	7·79*	7·85	7·93†	7·92
	Infected	22	7·57	7·73*	7·79†	7·84	7·86
	Infected	22·5	7·54	7·80*	7·78†	7·81	7·83
	Infected	24	7·48	7·64*	7·82†	7·85	7·88
C	Normal	22	7·46	7·68	7·72	—	—
	Normal	22	7·38	7·60	7·68	7·72	7·90
	Normal	22	7·38	7·51	7·59	7·68	7·72

Group A. Initial measurement of pH made on blood from flask.

Group B. Immediate measurement of pH made on blood put directly into glass electrode. In groups A and B the blood was diluted with 1 part of heparin-saline to 11 parts of blood.

Group C. Immediate measurement of pH made on blood put directly into glass electrode. Blood from chickens injected i.v. with 0·05 mg. heparin per 20 g. body weight.

* Emergence of the gametocytes first observed.

† Exflagellation first observed.

in different ampoules varied between 6·13 and 6·30, with a mean of 6·22. The buffering action of the blood presumably was sufficient to neutralize this difference in pH, but three series of measurements were made upon blood from normal chickens injected with heparin intravenously (Table 1, group C), for comparison with the measurements made upon blood to which heparin-saline had been added *in vitro*. Each chicken received 0·05 mg. of heparin per 20 g. body weight, by jugular vein, the total volume of fluid introduced being 0·05 ml. per 20 g. of body weight. Five minutes later 5 ml. of blood was removed from the jugular vein. One measurement of pH was made immediately, and the remainder of the blood was exposed to air in 1 ml. volumes, in Erlenmeyer flasks, and measurements of pH taken at 5 min. intervals as in previous experiments. In two birds, the immediate pH of the blood was 7·38 and in the third, 7·46. The final measurements made 20 min. later fell within the range of previous readings.

Although the variation in pH of blood in different flasks of a series was such that, in some series, the highest pH occurred in blood exposed to air for less than the maximum period, in the majority of experiments the pH of both normal and infected blood continued to rise throughout the 20 min. period.

V. THE RELATION OF THE pH OF THE BLOOD TO THE EMERGENCE OF THE GAMETOCYTES FROM THE ERYTHROCYTES, AND EXFLAGELLATION, *IN VITRO*

Attempts were made to determine the pH of the blood as soon as the gametocytes emerged from the erythrocytes or exflagellation occurred. In each of the eight experiments carried out on blood from different birds, heparinized blood in 1 ml. volumes was exposed to the air in a series of 25 ml. flasks. A drop of blood was removed from one of the flasks for microscopic examination, 5 min. after the blood had been withdrawn from the bird, and whilst it was being examined, the pH of the blood in the flask was determined. In none of the eight experiments were free gametocytes found immediately on examination of the blood, but they appeared 6–9 min. after the blood had been withdrawn from the bird, and were present on immediate examination of the sample from the second flask, i.e. 10 min. after the blood was withdrawn from the bird. The time of emergence of the gametocytes from the erythrocytes did not appear to be closely related to the pH of the blood. Free gametocytes were observed 1 min. after a sample of blood of pH 7·64 was removed from the flask, i.e. 6 min. after the withdrawal of the blood from the bird, whereas in a sample of blood of pH 7·79, no free gametocytes were seen until 4 min. had elapsed, i.e. 9 min. after the withdrawal of the blood from the bird. Since diffusion of CO_2, presumably, would continue from the blood at the edges of the cover-slip during the period of observation, an accurate measurement of pH at the precise moment the gametocytes emerged could not be made.

The temperature of the laboratory in which the experiments were carried out varied from 18 to 24° C. (Table 1), but no correlation was observed between the temperature and the time which elapsed between the withdrawal of the blood from the bird and the emergence of the gametocytes from the erythrocytes. In one of

two experiments in which the longest period (9 min.) elapsed between the withdrawal of the blood from the bird and the first appearance of free gametocytes, the temperature of the laboratory was 23° C., and in the other 21° C., whereas in another experiment in which only 7 min. elapsed, the laboratory temperature was only 18° C.

Samples of blood were removed from the remaining flasks of each series, at 5 min. intervals, and the pH of the remainder of the blood in the flask measured whilst the sample was examined microscopically for exflagellation. In eight experiments the shortest period from the time of withdrawal of the blood from the bird to the first appearance of exflagellation was 10 min. and the longest 21 min., the shortest period occurring at the highest temperature (24° C.) and the longest at the lowest (18° C.).

The lowest pH of blood in which exflagellating gametocytes were found was 7·78, but exflagellation did not necessarily occur immediately it had reached that level. In one experiment exflagellation was not seen until 6 min. after the pH had risen to 7·80. In another experiment in which the pH of the blood at the time of emergence of the gametocytes was 7·84, exflagellation was not seen until 5 min. after it had reached 7·93.

Owing to the continual rise in the pH of blood in which the gametocytes emerged from the corpuscles and exflagellated, it was not possible to correlate these processes with precise determinations of pH. An attempt was therefore made to stabilize the pH of the blood; the effect of buffer solutions upon the development of the gametocytes was accordingly studied.

VI. THE EFFECT OF TRIS BUFFERS UPON THE DEVELOPMENT OF THE GAMETOCYTES OF *PLASMODIUM GALLINACEUM*

In the following experiments Sigma 7–9 Biochemical buffer Tris (hydroxymethyl-aminomethane), with an approximate range of pH 7–9, made by Sigma Chemical Company, St Louis, U.S.A., was used. This buffer was chosen because parasitized erythrocytes suspended in isotonic solutions of it remained normal in appearance throughout the period of experiment, whereas in phosphate buffers some distortion occurred. A stock solution of Tris buffer, made up in NaCl solution so that the resulting buffer was isotonic with bird blood (121 mg. Tris + 965 mg. NaCl per 100 ml. glass-distilled water) was adjusted with N/10-HCl to give buffer solutions of the required pH. Heparinized, infected blood was mixed, immediately after it was withdrawn from the bird, with Tris buffer in the proportion of 0·4 ml. heparinized blood to 1·6 ml. of buffer, and exposed to air in a 25 ml. Erlenmeyer flask at the temperature of the laboratory. The times at which the gametocytes emerged and exflagellation took place were compared in the Tris-blood mixtures and in the controls, which in the first four experiments consisted of a similar volume of heparinized blood. As it was impossible to make a numerical comparison of the free and exflagellating gametocytes in the Tris-blood preparations and in those of the controls, the comparisons were based upon visual impressions formed by each observer after examining both preparations. The pH of the Tris-blood mixtures and of the controls was determined at the beginning of the experiment and as soon as exflagellation became frequent.

When parasitized blood was added to Tris buffer pH 8·12 in the above proportions, the pH of the mixture was lower than that of the buffer alone, but higher than that of the unbuffered blood (Table 2, Expts. 1 and 2). Although the pH of the Tris-buffer mixture rose slightly during the period of 15–20 min., it did not reach the original pH of the buffer. In the Tris-blood mixture the gametocytes emerged as soon as, or even earlier than, in the control, and exflagellating microgametocytes were numerous. The final pH, taken when exflagellating parasites were plentiful, was slightly higher in the Tris-blood mixture than in the control (Table 2, Expts. 1 and 2).

When parasitized blood was mixed with Tris buffer pH 7·20 (Table 2, Expt. 3), the initial pH was higher than that of the buffer alone, but not so high as that of the control. The final pH of the control, taken when exflagellation was frequent, was 7·82, but in the Tris-blood mixture it was slightly lower, i.e. 7·64, although owing to the slower development of exflagellation it was not determined until 10 min. later than in the control. In a second experiment with Tris buffer pH 7·20 (Table 2, Expt. 4), the initial pH of the Tris-blood mixture was slightly lower than in the previous experiment, and although observations were made at 5 min. intervals over a period of 30 min., few gametocytes emerged from the corpuscles, exflagellating parasites were rare and finally none could be found. The final pH, after exposure for 30 min., was 7·68.

In Expts. 1–4 a comparison of the frequency of exflagellating parasites in the Tris-blood preparations and in the heparinized blood was difficult, owing to the greater dilution of the former. Since the gametocytes emerged and exflagellating gametocytes appeared to develop as frequently and as quickly in blood mixed with Tris buffer pH 8·12 as in heparinized blood alone, 1:4 dilutions of blood in this buffer, or in Tris pH 8·22 were used as controls in later experiments (Table 2, Expts. 5–7).

When blood was mixed with Tris buffer pH 9·44 the initial pH (8·63) was lower than that of the buffer alone, and the pH fell during the 28 min. period of observation, but the final pH (8·37) was higher than in previous experiments or in the control. The higher pH had no adverse effect upon the development of the gametocytes; large numbers emerged from the corpuscles and exflagellation occurred at the same time and as frequently as in the controls.

Since the emergence of the gametocytes and exflagellation were partially inhibited when the pH of the blood was reduced by the addition of Tris buffer pH 7·20, attempts were made to inhibit these stages completely by reducing the pH of the blood further. When solutions of Tris, reduced to pH 4 and 3·37 by the addition of N-HCl, were added to parasitized blood in the proportions used in the previous experiments, the initial pH of the mixtures were 7·32 and 7·12 respectively owing to the buffering capacity of the blood, and the final pH readings, taken 25 and 45 min. after the blood was withdrawn from the body, were 7·65 and 7·47. Few gametocytes emerged from the corpuscles, and although exflagellation was not inhibited completely it was rare; the gametocytes and corpuscles were however normal in appearance (Table 2, Expts. 6 and 7).

Since Tris buffer pH 9·44 had no adverse effect upon the development of the

Table 2. *The effect of the* pH *of Tris buffer upon the emergence of gametocytes and exflagellation*

	Expt. 1. 20·5° C.		Expt. 2. 19·5° C.		Expt. 3. 20·5° C.		Expt. 4. 20·5° C.		Expt. 5. 21° C.		Expt. 6. 21° C.		Expt. 7. 21° C.	
	A	B	A	B	A	B	A	B	A	A	A	A	A	A
Buffer pH...	8·12	—	8·12	—	7·20	—	7·20	—	9·44	8·22	4·40	8·12	3·37	8·12
Initial pH...	7·85	7·60	7·82	7·57	7·47	7·58	7·40	7·50	8·63	8·00	7·32	7·90	7·12	7·80
Emergence (min.)														
0–5	.	.	+	.	.	.	.	.	.	.	.	.	.	.
5–10	+	+	.	+	.	.	.	+	.	.	.	+	(+)	+ +
10–15	.	.	.	.	.	+	(+)	.	+ +	+(+)	(+)	.	.	.
Exflagellation (min.)														
10–15	.	.	.	.	.	.	.	(+)	.	.	.	.	.	.
15–20	+ + +	+ +	+	+	.	+ +	(+)	+ +	+	(+)	(+)	+	(+)	+ +
	pH	pH				pH		pH				pH		pH
	7·97	7·86				7·82		7·78				7·97		7·87
20–25	.	.	+ + +	+ + +	.	.	+	.	+(+)	+ +	.	.	(+)	.
			pH	pH										
			7·93	7·90										
25–30	.	.	.	.	+	.	(+)	.	+ +	+ +	0	.	(+)	.
					pH				pH	pH			pH	
					7·64				8·37	7·90			7·47	
30–35	.	.	.	.	.	.	0	.	.	.	(+)	.	.	.
							pH							
							7·68							
35–40	.	.	.	.	.	.	.	.	.	.	(+)	.	.	.
40–45	.	.	.	.	.	.	.	.	.	.	0	.	.	.
											pH			
											7·65			

A = blood-buffer mixture; B = blood.

gametocytes, experiments were performed to discover whether the addition of Tris buffer of this high pH would reverse the inhibitory effect of buffer of low pH. 0·4 ml. of heparinized blood from a heavily infected chicken was added to each of four flasks, one of which contained 1·6 ml. Tris buffer pH 8·12 (Table 3, Expt. 1, flask A), and the remaining flasks (B, C and D) contained similar volumes of Tris buffer pH 3·37. The pH of the blood-buffer mixtures in flasks A and B were determined immediately, and drops of blood removed at intervals of 5 min. for microscopic examination. Large numbers of exflagellating gametocytes were found in flask A within 20 min., the pH being 7·80, but in flask B they were rare, the pH after 20 min. being 7·36. Twenty minutes after the beginning of the experiment, 2·0 ml. of Tris buffer pH 9·5 was added to flask C, and, in order that the volume of liquid in the two flasks should be equal, 2·0 ml. of Tris buffer pH 3·37 was added to flask D. In the examinations made during the first 10 min. after the addition of Tris buffer pH 9·5 few exflagellating gametocytes were found in flask C, though the gametocytes were emerging from the corpuscles, but within 25 min. exflagellating gametocytes were as numerous as they had been in flask A; the pH in flask C was 8·23. In flask D few exflagellating microgametocytes were seen during examinations covering a period of 25 min., the final pH being 7·13. A second and third experiment with Tris buffer pH 3·25 gave similar results, but although the final pH of the Tris-blood mixture in flask D was only 6·93 in one experiment and 6·75 in the other, exflagellating microgametocytes were occasionally found (Table 3, Expts. 2 and 3). In a fourth experiment, however, the addition of Tris buffer pH 9·5 to the mixture of Tris buffer pH 3·25 and blood failed to stimulate the gametocytes, although the pH after 26 min. was 8·19, whereas in blood from the same source treated with Tris buffer pH 3·25 (Table 3, Expt. 4) exflagellation was slightly more frequent, although the final pH was only 6·96. Thus the addition of alkaline buffer does not always reverse the inhibitory effect of low pH upon the development of the gametocytes.

The observation that buffer of low pH inhibited the development of the gametocytes considerably but not completely, whereas CO_2 inhibited their development completely, suggested that CO_2 might have some action in addition to its effect upon the pH of the blood. Isotonic Tris buffer pH 9·44 was exposed to 5% CO_2 + 95% O_2 for 10 min. and repeatedly shaken, as in the experiments described in section III. Blood from a heavily infected bird was added in the proportion of 1 to 4 parts of buffer, and the mixture exposed to the gas for 20 min. In samples of the mixture removed at 5 min. intervals during this period, gametocytes emerged from the erythrocytes and exflagellating microgametocytes were as numerous in the final sample as in a sample of the control, which consisted of a similar mixture of blood and buffer exposed to air for a similar period. The pH of the blood-buffer mixture after exposure to 5% CO_2 + 95% O_2 for 30 min. was 7·62, whereas that of the control was 8·56. In another experiment in which 5% CO_2 + 95% O_2 was passed through Tris buffer pH 9·44 for 60 min. before the parasitized blood was added, no gametocytes emerged from the corpuscles and no exflagellation occurred during 35 min. exposure to the gas, but the pH at the end of this period had fallen to 7·20. In the control, consisting of a similar blood-buffer

Table 3. *The reversible effect of* pH *on exflagellation*

	Expt. 1. 21·5° C.				Expt. 2. 20·5° C.				Expt. 3. 22° C.				Expt. 4. 21·5° C.			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Buffer pH...	8·12	3·37	3·37	3·37	8·12	3·25	3·25	3·25	8·22	3·25	3·25	3·25	8·12	3·25	3·25	3·25
Initial pH...	7·67	7·10	.	.	7·62	6·99	.	.	8·10	7·10	.	.	7·67	7·08	.	.
Exflagellation (min.)																
10–15	+	(+)	.	.	(+)	(+)	.	.	(+)	(+)	.	.	(+)	(+)	.	.
15–20	+++	(+)			+++	(+)			++	0			+++	(+)	.	.
	pH 7·80	pH 7·36	pH* 9·5	pH† 3·37			pH* 9·5	pH† 3·25			pH* 9·5	pH† 3·25	pH 7·87	pH 7·32	pH* 9·5	pH† 3·25
20–25	.	.	(+)	(+)	+++	(+)	0	(+)	++	(+)	.	.	.	.	(+)	(+)
					pH 7·78	pH 7·33			pH 8·07	pH 7·50						
25–30	.	.	(+)	(+)	.	.	(+)	(+)	.	.	0	0	.	.	(+)	(+)
30–35	.	.	++	(+)	.	.	(+)	(+)	.	.	(+)	(+)	.	.	(+)	+
35–40	.	.	+++	(+)	.	.	(+)	(+)	.	.	(+)	0	.	.	(+)	+(+)
			pH 8·23	pH 7·13												pH 6·96
40–45	.	.	.	.	.	.	(+)	0	.	.	+	(+)	.	.	(+)	.
															pH 8·19	
45–50	.	.	.	.	.	.	(+)	0	.	.	++	0	.	.	+	.
											pH 8·40	pH 6·75				
50–55	.	.	.	.	.	.	+++	(+)	.	.	.	.	.	.	(+)	.
							pH 8·10	pH 6·93								

* = 2·0 ml. Tris buffer pH 9·5 added.

† = 2·0 ml. Tris buffer pH 3·37 or 3·25 added.

For explanation of A, B, C, & D, see text.

mixture exposed to air, exflagellation occurred within this period, the pH being 8·54. CO_2, therefore, failed to inhibit the development of the gametocytes when the pH was 7·62, though it produced complete inhibition when the pH was allowed to fall to 7·20. In this experiment in which the development of the gametocytes was completely inhibited by CO_2, the pH of the blood was higher than in the experiments in which blood was diluted with the buffer of low pH (Table 3, Expts. 1–4, flasks D), yet in these latter experiments inhibition was not complete.

When parasitized blood was added to Tris buffers in the proportion of 1 part to 4 its buffering action affected the pH of the mixture. The effect of Tris buffers upon washed corpuscles was therefore studied. 1 ml. of heparinized blood rich in gametocytes was thoroughly mixed with 4 ml. of Tris buffer pH 8·20, and the parasitized corpuscles separated from the liquid by centrifugation. The supernatant fluid was removed and the parasitized corpuscles resuspended in 5 ml. of the buffer. Exflagellation occurred within 20 min. in a sample of heparinized blood, from the same bird, exposed to the air in a flask. No exflagellation was seen in samples of the washed, resuspended corpuscles over a period of 40 min., though the suspension was exposed to the air in a thin layer as in the control. The buffer suspension of corpuscles was then divided into two equal volumes and centrifuged once more. After removing the supernatant, the corpuscles in one tube were resuspended in fresh buffer, whereas in the other they were resuspended in fresh normal plasma. In the tube containing buffer, no gametocytes emerged from the corpuscles over a period of 50 min., the final pH being 7·93, but in the tube containing plasma, exflagellation began within 14 min. of the addition of the plasma and gradually became frequent. The pH immediately after the addition of the plasma was 7·65 and rose, during the 50 min. period of observation, to 7·86.

This experiment was repeated with isotonic Tris buffer pH 7·78. As in the previous experiment the gametocytes failed to emerge from the washed corpuscles, and no exflagellation took place in the buffer. The corpuscles from one tube were resuspended in normal chick plasma and exposed to air as a thin layer, the buffer suspension from the second tube likewise being exposed to air in a flask. Within 8 min. of the addition of plasma, gametocytes began to emerge from the corpuscles and exflagellation occurred 9 min. later. No exflagellation occurred in the buffer preparation, nor did the gametocytes emerge from the corpuscles. The pH of the plasma suspension was 8·32 and that of the buffer suspension, 7·72.

Centrifugation alone was found to have no adverse effect upon either the time or frequency of exflagellation.

Since exflagellation occurred readily in infected blood mixed with Tris buffer to give a pH of 7·8 or higher, but not in washed, infected corpuscles suspended in buffer to give a similar pH, it must be concluded that the pH of the fluid in which the parasitized corpuscles are suspended is not the only factor controlling the emergence of the gametocytes from the corpuscles or exflagellation of the microgametocytes: some factor present in plasma also seems to be necessary.

VII. THE EFFECT OF DIAMOX UPON THE pH OF CHICKEN BLOOD AND UPON THE DEVELOPMENT OF THE GAMETOCYTES OF *PLASMODIUM GALLINACEUM*

The enzyme carbonic anhydrase, which catalyses the reversible reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, was shown by Mann & Keilin (1940) to be inhibited by sulphonamides. More recently, Diamox (2-acetylamino-1,3,4-thiadiazole-5-sulphonamide), synthesized by Roblin & Clapp (1950), was shown to be 330 times as effective an inhibitor as sulphanilamide (Miller, Dessert & Roblin, 1950). Since carbonic anhydrase has been shown to play an important part in CO_2 transport in the blood, it was thought that its inhibition might prevent or delay the emergence of the gametocytes from the erythrocytes and the subsequent exflagellation of the male gametocytes.

In the first experiment Diamox (12·5 mg./20 g. of body weight of bird) was given orally, as a suspension in water, to heavily infected chicks whose blood contained large numbers of mature gametocytes. When 1 ml. of freshly drawn, heparinized blood, withdrawn from the bird 2 hr. after the administration of the drug, was exposed to air in a 25 ml. flask, the gametocytes emerged normally and exflagellation occurred within 20 min. In a control sample, taken from the bird before administration of the drug, exflagellation also occurred within 20 min. In a second experiment the gametocytes emerged from the erythrocytes and exflagellation took place after the same period both in the control sample, taken before the administration of the drug, and in blood taken 4 hr. after the bird had received 27 mg. of Diamox per 20 g. of body weight. This dose produced some slight symptoms of toxicity in the bird.

Since Diamox given orally was without effect upon the gametocytes, its effect when given intravenously was studied. The drug was dissolved in the minimal amount of NaOH necessary for complete solution. In the first experiment the pH of the solution was 8·17. Forty-five minutes after the bird had received a dose of 1 mg. of Diamox per 20 g. body weight, the carbonic anhydrase activity of the blood was tested, by the method of Philpot & Philpot (1936), and found to be almost completely inhibited. The immediate pH of a sample of blood withdrawn at this time was 7·19. Seven minutes after the sample of blood had been withdrawn from the body the gametocytes began to emerge from the corpuscles, and 9 min. later exflagellating gametocytes were observed and became as frequent as in the control sample of blood taken from the bird before the administration of the drug.

In a second experiment a heavily infected bird was given a similar dose of Diamox (pH 8·68) intravenously and bled 15 min. later. The immediate pH of the blood was 7·38 and exflagellation occurred 15 min. after the exposure of the blood to air, the pH having risen to 7·56. A second sample of blood was removed from the bird 1 hr. after the administration of the drug, the immediate pH being 7·30. Exflagellation again occurred after 15 min., the pH being 7·55. Sixty-five minutes after the first dose was administered, a second similar dose of Diamox was given and a sample of blood removed from the bird 37 min. later. The immediate pH of this sample was 7·12. After 16 min. exposure of the blood to air exflagellating

gametocytes were numerous, though the pH of the blood was only 7·22. In this experiment the pH of the blood during the period in which the gametocytes were developing was below that of blood immediately it is removed from an untreated infected bird. In a third experiment two doses of Diamox (1 mg./20 g. body weight, pH 8·49) were given at an interval of 35 min. Forty-five minutes after the second dose a sample of blood was removed and the immediate pH found to be 7·08. Twenty minutes later, after exposure to air, gametocytes were emerging from the corpuscles, and 5 min. later exflagellating microgametocytes were seen, though the pH had risen to only 7·41.

In a further similar experiment in which two doses of Diamox were given at an interval of 30 min., the immediate pH of the blood, 30 min. after the second dose, was 7·22. Free gametocytes were seen within 10 min. of the sample of blood being withdrawn from the bird, and exflagellation was observed after a further period of 10 min., the pH of the blood at the time when exflagellation was well established being 7·46.

VIII. A COMPARISON OF THE TIME TAKEN FOR THE DEVELOPMENT OF EXFLAGELLATION OF MICROGAMETOCYTES OF *PLASMODIUM GALLINACEUM IN VITRO* AND IN THE STOMACH OF *AËDES AEGYPTI*

From their observations upon *P. elongatum*, *P. relictum* and *P. cathemerium* in the stomach of *Culex pipiens*, *C. quinquefasciatus*, *Aëdes aegypti* and *Anopheles quadrimaculatus*, Micks *et al.* (1948) concluded that there is a chemical factor, present in the stomach secretions of susceptible mosquitoes, which is responsible for stimulating the parasite to exflagellate and complete fertilization, whereas in insusceptible species there is a factor which inhibits the activities of the gametocytes almost completely. Since the addition of stomach contents from unfed *Culex pipiens* to infected blood did not induce exflagellation, the authors concluded that the taking of a blood meal is necessary to the production of these secretions.

If some factor stimulating the development of the gametocytes is produced by the mosquito during ingestion of the blood, development should be more rapid in blood from the stomachs of recently engorged mosquitoes than in blood withdrawn directly from the infected bird. A comparison was therefore made of the time taken for the development of exflagellation in 1 ml. of parasitized blood exposed to the air as a thin layer, and in the stomachs of *Aëdes aegypti* fed upon the same bird.

In the following experiments the birds were injected intravenously with heparin in saline to prevent coagulation of the blood. Small gauze-covered containers, each containing about six female mosquitoes, were applied to the bird and were removed as soon as one or more of the mosquitoes were fully engorged. Five to 20 min. later, the mosquitoes were lightly anaesthetized with ether, the rear end of the abdomen was punctured with a sharp needle, and a film was made without delay from the blood which flowed out from the stomach. The stages of development of the gametocytes were studied in the stained films. Alternatively, the drop of blood was immediately covered with a cover-slip and observed under the microscope for 3 min. Since the process of engorgement may take several minutes to complete, it is

impossible to time the onset of exflagellation as accurately in blood from the mosquito's stomach as in blood withdrawn from the bird by syringe.

In the first experiment exflagellating gametocytes were first seen in blood from mosquitoes 12 to 13 min. after the insects had engorged. In blood withdrawn from the same bird and maintained *in vitro* exposed to the air in a thin layer, exflagellation occurred in 12 min. In a second experiment exflagellating gametocytes were found in two mosquitoes killed 14 min. after the blood meal, and in one mosquito killed 15 min. after engorgement. Exflagellation was not observed in mosquitoes killed earlier than 14 min. after the completion of feeding, but in all mosquitoes killed after 15 min. or longer exflagellating gametocytes were numerous. In blood obtained by syringe from the chicken on which the mosquitoes had engorged and exposed as a thin film to air, exflagellation occurred in 16 min.

These experiments are open to the criticism that the conditions in the mosquitoes' stomachs were abnormal as the blood was prevented by heparin from coagulating. However, in mosquitoes fed upon a bird free from heparin, exflagellation was first seen 13 min. after the mosquito had fully engorged, and in blood taken from the same bird by syringe, exflagellation occurred in 12 min.

No significant difference, therefore, was observed between the time taken for exflagellation to develop in mosquitoes' stomachs and in blood taken from the same bird by syringe.

The effect of the addition of crushed salivary glands or stomachs of unfed *A. aegypti* to heparinized infected blood was also studied. In such experiments it was most important that the length of time during which the drops of blood were exposed to the air before the cover-slips were put into place should be the same, both in the drops containing the crushed stomachs or salivary glands and in the control drops, as a slight difference in the time of exposure to air could affect the results significantly. Thus it was found that in drops of blood exposed to the air for 3 min. before the cover-slips were applied and sealed with wax, exflagellating gametocytes developed in large numbers 10 or more minutes later, whereas in drops from which the air was immediately excluded they were very rare and occurred only in very restricted areas of the preparation.

In testing the effect of crushed stomachs or salivary glands, the stomachs or glands in saline were added to a drop of parasitized blood rich in gametocytes and rapidly mixed together, and an equal volume of saline was added to and mixed with a control drop. The cover-slips were put into place simultaneously and sealed. In preparations made in this manner the addition of salivary glands or stomachs was without effect upon the time or frequency of occurrence of exflagellation.

IX. MEASUREMENTS OF THE pH OF BLOOD FROM THE STOMACHS OF *AËDES AEGYPTI* FED UPON CHICKENS

Since there was no difference in the time taken for the gametocytes of *P. gallinaceum* to develop in the stomach of *A. aegypti* fed upon infected birds and in infected blood withdrawn by syringe and exposed to air, it was of interest to know whether the pH of the blood in the mosquitoes' stomachs was the same as that of

blood in which these changes were taking place *in vitro*. The pH of blood from mosquitoes' stomachs was therefore compared with that of blood withdrawn directly from the birds. In making these measurements a modification of the micro-glass electrode described by Caldwell (1954) was used (Bishop *et al.* 1956).

The mosquitoes were fed upon birds which had previously been injected intravenously with 0·05 mg. heparin per 20 g. of body weight, and only those which were fully distended with blood were used. Immediately before the blood was collected the mosquitoes were immobilized by light anaesthetization with chloroform or by exposing them, for a few minutes, to a temperature slightly above freezing. Several mosquitoes were fixed dorsally to a microscope slide by a drop of paraffin wax, and the operation of collecting the blood carried out under a binocular microscope. The abdomen was punctured by a very fine hard-glass capillary, the stomach was penetrated and the blood collected directly by capillarity. In some cases it was possible to collect sufficient blood for a measurement from a single mosquito, but in others it was necessary to collect blood from two or more stomachs. For comparison, blood was collected in capillary tubes directly from the jugular vein of a chicken and the pH measured immediately. With young chickens no difficulty was experienced in penetrating the jugular vein with the pointed end of the capillary. Two readings were taken, at intervals of 60 sec., upon each sample of blood, a reading upon a standard buffer pH 7·00 being taken, in a similar capillary tube with the same micro-glass electrode, immediately before and after the readings upon the blood.

The pH of each of five samples of blood taken directly from the jugular vein of chickens was 7·51, the lowest reading of the standard buffer being 6·98 and the highest 7·01. The pH of blood taken by this method falls within the range of the immediate pH of chick blood obtained by the Hartree micro-glass electrode (Table 1).

Blood was removed from the mosquitoes' stomachs after varying periods following engorgement. In each experiment the mosquitoes, enclosed in a gauze-covered container, were applied to the chicken for the minimum time required for several mosquitoes to become fully engorged; but, as the speed with which they began to gorge and the rapidity of feeding varied, it was impossible to determine accurately how long the blood had been in the stomach at the time of collection. In comparing the effect of the time the blood had been in the mosquito's stomach upon the pH (Table 4), the period between the application of the container to the chicken and the collection of the blood from the mosquito's stomach has been divided into two sections, a period of engorgement during which the mosquitoes had access to the bird, and the period between their removal from the bird and the collection of the ingested blood.

The lowest pH recorded of blood from mosquitoes' stomachs was 7·47 and the highest 7·90, but all except six of the thirty readings were between 7·60 and 7·76. Since the standard error of pH measurements made with the micro-glass electrode was less than 0·05 (Bishop *et al.* 1956), these differences must be due to real differences in the pH of the ingested blood and not to errors in the electrode readings. The mean value, pH 7·68, was higher than that of blood taken

directly from the chickens (pH 7·51) and measured by this method. The mean pH of blood from mosquitoes' stomachs is also higher than the immediate pH of chicken blood as measured in the Hartree micro-glass electrode, but falls within the range of pH attained by blood after exposure to air; unlike drawn blood, in which the pH rises during exposure to air for 20 min., no increase in pH was detected in blood in the mosquito stomach, over a period of 1 hr. after engorgement. Although successive readings could not be made on blood from a single mosquito, there was

Table 4. *Measurements of* pH *of blood from stomachs of* Aëdes aegypti

Period of engorgement	Period between end of engorgement and collection of blood	No. of mosquitoes	pH of blood*	Error of standard reading (pH 7)
4	17	1	7·60	−0·03
4	23	1	7·70	0
4	31	2	7·60	+0·03
4	38	1	7·55	+0·01
5	6	5	7·82	0
5	7	4	7·87	+0·02
5	7	3	7·65	0
5	8	4	7·71	+0·02
5	9	3	7·73	−0·02
5	10	2	7·72	0
5	14	2	7·62	0
5	14	3	7·77	0
5	20	3	7·76	+0·01
5	21	4	7·62	+0·02
5	28	3	7·62	0
5	30	2	7·60	+0·01
5	31	2	7·47	+0·02
5	54	2	7·67	+0·01
5	64	3	7·73	−0·03
5	70	3	7·58	−0·01
6	3	1	7·69	0
6	7	3	7·66	+0·01
6	10	2	7·63	+0·02
6	15	6	7·68	−0·01
6	33	2	7·68	+0·02
6	48	3	7·70	+0·02
6	66	4	7·76	−0·01
7	7	5	7·90	−0·01
10	33	3	7·71	0
15	5	1	7·60	−0·03

* Mean of two readings on the same sample taken at intervals of 60 sec.

no correlation between the pH of the ingested blood and the time it had been in the mosquito's stomach. In some cases, the pH of blood which had been in the mosquito's stomach for at least 30 min. was lower (e.g. pH 7·60) than that which had been ingested approximately only 6 or 7 min. before the measurement was made (e.g. pH 7·82 and 7·90).

No significant difference in pH could be found between blood from single mosquitoes and pooled blood from several, though the highest measurements were made upon pooled blood.

X. DISCUSSION

The gametocytes of plasmodia do not emerge from the erythrocytes whilst in the blood stream of the vertebrate host, but if the blood is ingested by susceptible mosquitoes, or withdrawn artificially and exposed to air, they emerge and the male gametocyte proceeds to exflagellate. In *Plasmodium gallinaceum* the time required for these processes to take place is approximately the same, whether they occur in the mosquito's stomach or in blood exposed to air as a thin layer. The fact that few gametocytes emerge from the erythrocytes and that exflagellation is rare if blood rich in gametocytes is sealed under a cover-slip immediately after its withdrawal from the bird, whereas these stages are numerous if a drop of infected blood is exposed to air for as short a period as 3 min., indicates that the factor or factors which produce these changes begin to act immediately, or almost immediately, after the blood is withdrawn from the body, although the gametocytes do not emerge from the erythrocytes until some minutes later.

Marchoux & Chorine's (1932) observation, that the emergence of the gametocytes of *Haemoproteus* from the erythrocytes and the exflagellation of the male gametocytes could be inhibited completely and reversibly by CO_2, has been confirmed in *P. gallinaceum*, although in our experiments satisfactory recovery could be obtained only after exposure to 5 % CO_2 + 95 % O_2 or 5 % CO_2 + 95 % N_2, and not after exposure to pure CO_2 as used by Marchoux & Chorine. The time taken for the gametocytes to emerge from the erythrocytes and for exflagellation of the male gametocytes to take place, was approximately the same after the 5 % CO_2 + 95 % O_2 had been replaced by air as in freshly drawn blood exposed to air as a thin layer, or in blood newly ingested by mosquitoes.

Marchoux & Chorine found that the emergence of the gametocytes of *Haemoproteus* and exflagellation proceeded normally if the pH of the blood was adjusted to 7·6, but was inhibited by a pH below that of normal blood, and they concluded that a rise in pH, due to diffusion of CO_2 from the blood, is responsible for the changes in the gametocytes when parasitized blood is withdrawn from the vertebrate host. Although we have found that the pH of freshly drawn blood heavily infected with *P. gallinaceum* begins to rise as soon as it is exposed to air, and continues to rise during a 20 min. period of observation—a period during which gametocytes emerge from the erythrocytes and exflagellation takes place—we have not found it possible to inhibit the development of the gametocytes completely by buffering blood to a pH below that of freshly drawn chicken blood; complete inhibition, such as is obtained by exposure of the blood to 5 % CO_2, did not occur, though few gametocytes emerged and exflagellated. In a blood-buffer mixture exposed to 5 % CO_2 + 95 % O_2, however, the development of the gametocytes was inhibited completely though the pH was higher (7·20) than that of blood-buffer mixtures in which inhibition was not complete (6·75–7·13, Table 3); but when the pH of the blood-buffer mixture exposed to the gas did not fall below 7·62, the gas failed to inhibit their development.

Evidence that pH alone is not sufficient to inhibit the emergence of the gametocytes from the corpuscles and their further development was obtained from birds

injected intravenously with Diamox. In blood withdrawn from one bird treated with this drug, the pH at the time when large numbers of gametocytes were exflagellating (pH 7·22), was below the lowest pH recorded for blood immediately after its withdrawal from an untreated bird (7·33); as the immediate pH of the blood from the Diamox-treated bird (pH 7·12) had been even lower, the whole process of development leading to exflagellation must have taken place at a pH significantly below that of the blood stream of a bird which had not been treated with the drug. In two other experiments the immediate pH of the blood was significantly lower than that of untreated birds, and at the time when exflagellating gametocytes were numerous the pH was still within the range of readings of the immediate pH of blood from untreated birds. In these two birds, also, the development of the gametocytes must have begun whilst the pH of the blood was below that of the untreated birds. It could be suggested that any change in the pH of the blood, either above or below the normal, might initiate these changes in the gametocytes: if this were so, free gametocytes and exflagellating male gametocytes would have been present in the freshly drawn blood of Diamox-treated birds; no change in the gametocytes was observed, however, in the blood of Diamox-treated birds until after approximately the same period as in blood from untreated birds.

In comparing the non-inhibitory effect of low pH upon the development of the gametocytes in blood from Diamox-treated birds with its partially inhibitory action in blood-buffer mixtures (Tables 2 and 3), a factor other than pH must be considered—the dilution of the blood. In the experiments with buffers, the blood was diluted 1:5 or 1:9 with the buffer, whereas the blood from Diamox-treated birds was undiluted. That the dilution of the plasma used in these experiments was not intrinsically inhibitory was shown by the fact that the partial inhibition could be reversed by the addition of buffer of a higher pH, which involved further dilution of the blood; but it is possible that in diluted plasma the gametocytes were more sensitive to a pH below the normal.

The possibility that Diamox stimulated the development of the gametocytes by some mechanism not present in untreated birds, and that results from birds treated with this drug are not comparable with those from untreated birds, cannot be excluded.

Micks *et al.* (1948) found the mean pH of canary blood to be 7·61 and that of duck 7·65. The mean pH of blood from the stomachs of *Culex pipiens*, *C. quinquefasciatus* and *Aëdes aegypti* fed upon duck's blood was 7·52, 7·60 and 7·67 respectively. As several of the individual measurements from the two former species, in which *P. elongatum* develops, were lower than the mean measurements for blood taken direct from the canary or duck, they concluded that there was no correlation between pH and exflagellation. With one exception, all our measurements of the pH of blood from the mosquito's stomach were higher than those of freshly drawn chick blood made by the same method, but these latter measurements (pH 7·51) were lower than those of duck or canary blood obtained by Micks and his colleagues. However, the pH of blood from several of the mosquitoes' stomachs, 30 min. after engorgement—a period during which emergence of the

gametocytes and exflagellation presumably had taken place—was lower than that of exposed blood, as determined by the Hartree micro-glass electrode, in which free gametocytes were present.

No evidence was obtained, from our measurements of the pH of blood from mosquitoes' stomachs, of a gradual rise in pH similar to that observed in drawn blood exposed to air. Although a difference in pH of 0·43 was observed between the highest and lowest measurements, this could not be correlated with the time since engorgement. The question therefore arises whether CO_2 diffuses from the ingested blood in the mosquito's stomach at a rate comparable to that of a similar volume of blood exposed to air. The numerous tracheoles present on the stomach walls suggest that diffusion of CO_2 from ingested blood may take place rapidly, but no data are available. In the mosquito's stomach, however, the pH of the blood may be affected by factors not present in drawn blood. The time taken for the gametocytes to emerge from the corpuscles and the male gametocytes to exflagellate is the same in blood exposed to air and in blood in the mosquito's stomach, which suggests that the inducing factors are the same or closely related, but so little is known of the digestive processes in the mosquito that it is, at present, only profitable to consider the conditions under which the gametocytes develop in drawn blood.

The data from the experiments on drawn blood do not suggest that pH is the critical factor inducing the development of the gametocytes. The suggestion tentatively offered in explanation of our results is that a factor, or factors, is present in the plasma which is activated, directly or indirectly, by diffusion of CO_2 from the blood as soon as it is exposed to air. In whole blood from birds treated with Diamox the factor appears to be independent of pH, but in diluted blood the development of the gametocytes appears to be partially dependent upon pH.

XI. SUMMARY

1. The emergence of the gametocytes of *Plasmodium gallinaceum* from the erythrocytes and the exflagellation of the male gametocyte were inhibited completely when infected blood was exposed to an atmosphere of 5 % CO_2 and 95 % O_2, or 5 % CO_2 and 95 % N_2. When these gas mixtures were replaced by air, the gametocytes emerged from the erythrocytes and the male gametocytes exflagellated after approximately the same interval as they did in newly drawn blood exposed to air, or in blood ingested by mosquitoes. If pure CO_2 were used instead of the gas mixtures containing 5 % CO_2, the development of the gametocytes, after the replacement of the gas by air, was uncertain.

2. The emergence of the gametocytes and exflagellation of the male gametocytes proceeded normally when parasitized blood was exposed to an atmosphere of N_2.

3. The pH of freshly drawn normal chick blood was 7·38–7·46. The pH of blood exposed to air rose over a 20 min. period of observation, the highest pH recorded being 7·97. No significant difference in pH was observed between normal and heavily infected blood. It was not possible to correlate the stages of development of the gametocytes with precise values of pH of the blood.

4. The development of the gametocytes proceeded normally when parasitized blood was mixed with isotonic alkaline Tris buffer to give a pH of approximately 8·0; but it was inhibited, though not completely, when the pH was reduced below that of freshly drawn blood by the addition of isotonic acid buffer. The inhibitory effect of the acid buffer could be completely but not invariably reversed by alkaline buffer.

5. 5 % CO_2 had no inhibitory action upon the gametocytes if the pH of the blood-buffer mixture was 7·62, but when it was 7·20 the inhibitory effect of the gas was complete.

6. The emergence of the gametocytes from the corpuscles and the exflagellation of the male gametocytes were completely inhibited when washed, parasitized corpuscles were suspended in isotonic buffer pH 7·78 or 8·20, but this inhibition was completely reversed when the corpuscles were resuspended in plasma. It is concluded that a factor present in plasma is necessary for the development of the gametocytes.

7. Further evidence that the pH of the blood, alone, is not the only factor affecting the emergence of the gametocytes and exflagellation of the male gametocytes was obtained from birds treated intravenously with Diamox. When blood from such birds was exposed to air the gametocytes emerged from the corpuscles and exflagellation took place, although the pH of the blood was as low as, or lower than, that of freshly drawn blood.

8. The emergence of the gametocytes from the erythrocytes and exflagellation of the male gametocytes were not stimulated by secretions from the stomachs or salivary glands of gorged or unfed mosquitoes.

9. The lowest measurement of pH of blood from mosquitoes' stomachs was 7·47 and the highest 7·90, the mean being 7·68, which is slightly higher than that of freshly drawn chick blood. No correlation could be found between the pH of the blood and the length of time since its ingestion by the mosquito.

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A METHOD FOR DETERMINING THE pH OF SMALL QUANTITIES OF FLUID

By ANN BISHOP, E. F. HARTREE and ELSPETH W. McCONNACHIE

(With 1 Figure in the Text)

The micro-glass electrode used in measuring the pH of blood from the stomachs of mosquitoes was a modification of that described by Caldwell (1954). The electrode was made of capillary tubing, drawn from Corning 015 glass, the outer diameter being 0·5–0·75 mm. and the length about 6 cm. As the electrode was used with a Pye Universal pH meter, and not with the electrometer impedance converter circuit used by Caldwell, it was important that the wall of the capillary tube should be very thin, in order that the resistance of the electrode should not exceed 500 MΩ, the maximum electrode resistance permissible with the Pye Universal pH meter. The capillary tube was coated with melted shellac, leaving 1·0–1·5 cm.

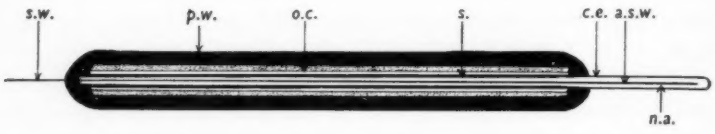

Fig. 1. *s.w.*, silver wire; *p.w.*, picene wax insulation; *o.c.*, outer capillary; *s.*, shellac covering of capillary; *c.e.*, capillary of electrode glass; *a.s.w.*, anodized portion of silver wire; *n.a.*, N/10 hydrochloric acid.

of the tube at one end and a shorter length at the other end uncoated. The coated capillary tube was passed into a protective, hard glass, thick-walled capillary tube of slightly greater diameter, its length being such that about 1·0–1·25 cm. of the inner capillary tube projected at one end and about 0·25 cm. at the other. The electrode was insulated by covering the outer tube and sealing its junctions with the inner tube, with heated picene wax, care being taken to leave at least 0·75 cm. of the inner tube exposed at one end and about 0·2 cm. at the other. The inner capillary tube was filled with N/10-HCl by capillarity, and the longer end was sealed by heating in a small flame. A silver wire, 0·1 mm. in diameter and about 12 cm. in length, was anodized in N/10-HCl for about one-fifth of its length. The anodized portion was passed down the capillary tube until its distal end almost reached the sealed end of the tube. The open end of the capillary tube, from which the unanodized portion of the silver wire projected, was sealed with shellac, and the picene wax insulation extended to cover this end of the capillary tube (Fig. 1). Air bubbles at the sealed end of the electrode were removed by centrifuging at a low speed. The resistance of the electrodes was determined before use and only those with a resistance below 500 MΩ were retained. The exposed capillary tube of the electrode was soaked in N/10-HCl for 24 hr. before the electrode was calibrated. When the

electrode was ready for use, the projecting silver wire was soldered to a screened lead for connexion with the meter.

The calomel electrode assembly used with the micro-glass electrode was made in the following way. A polythene tube, 1·5 cm. in external diameter, was heated and drawn out so that it tapered to form a thin-walled tube about 1·5 mm. in external diameter at its distal end. A little hot agar-KCl (Cole, 1933) was drawn into the tip of the capillary and allowed to solidify to form a plug. The polythene tube was then filled with solid KCl plus a saturated solution of KCl to cover the end of the calomel electrode (Cambridge Instrument Co.), which was fixed into the upper and wider end of the polythene tube through a rubber bung. Two small holes, bored through the polythene tube above the level of the solution, allowed the air to escape when the rubber bung holding the calomel electrode was pushed into place. The advantage of this polythene tube over a similar pattern in glass, was that it was flexible and unbreakable. The readings obtained with it were much steadier than those obtained when a cotton thread soaked with saturated KCl was used as a bridge—a method sometimes used in pH determinations on small volumes of fluid.

For measurement of pH, the fluids were collected in hard glass capillaries having an internal diameter slightly wider than that of the exposed capillary of the electrode. The end of the capillary was drawn to a fine point, care being taken that the tip remained open so that fluid could enter. In collecting blood from mosquitoes or from birds, capillaries 4–7 cm. in length were used for ease in handling. When sufficient blood had been collected, the capillary was cut through about 1·0–1·5 cm. from the tip, thus forming a capillary container with a pointed open end. The capillary containers were handled with forceps so that their contents did not become warmed by contact with the fingers of the operator.

The agar-KCl bridge into which the tips of the capillary containers and the capillary end of the polythene sheath of the calomel electrode were thrust, was made of 5% agar to give a stiff gel in which the containers would stand upright. The agar-KCl gel was contained in a Perspex trough (internal dimensions 5·25 × 5·25 × 1·0 cm.) fitted into the mechanical stage of a microscope from which the barrel had been removed. The capillary containers sticking in the agar-KCl gel could thus easily be moved into position beneath the micro-electrode by means of the mechanical stage.

For lowering the micro-glass electrode into the capillary containers, the focusing mechanism of a small monocular dissecting microscope was used. In order to obtain the required height, a vertical extension was made to the adjustable column which normally carries the lens. The micro-electrode was fixed vertically in a slotted holder made of polythene tubing, and the holder was held in a clamp attached, in a horizontal position, to the vertical extension of the dissecting microscope. The delicate adjustments necessary for centring the capillary container under the micro-electrode, and for lowering the electrode until its exposed capillary was immersed in the fluid, were carried out with the aid of a large lens, fixed in a stand, through which observations upon the relative positions of the electrode capillary and the capillary containers could be made with greater ease than with the naked eye. It was found necessary to immerse the glass capillary of the

electrode completely, i.e. as far as the insulation, in order to obtain consistent readings. By this method it was possible to make a reading upon as small a volume of fluid as 0·005 ml.

The calomel electrode in its polythene tube was clamped in such a position that the tip of the polythene tube dipped into the agar-KCl in the trough. The flexible nature of the tube made further adjustments unnecessary.

Capillary containers filled with glass-distilled water or buffer, were used for washing the capillary glass electrodes. The capillary was washed between each reading, and dried with absorbent tissue.

Not all electrodes with a resistance less than 500 MΩ gave consistent readings nor could the degree of accuracy shown by individual electrodes be correlated with resistance.

An electrode was considered suitable for use if successive determinations of the pH of known buffers gave reasonably consistent results. None of the capillary glass electrodes constructed produced the expected deflexion of the pH meter when used with previously standardized buffers, and each electrode had, therefore, to be calibrated before it could be used for determining the pH of blood.

The pH values of a series of Sørensen phosphate buffers, pH 6·18–8·01, were determined using the Hartree micro-glass electrode (Hartree, 1952) the standard being a phosphate solution pH 7·00. Each of the capillary electrodes was then calibrated as follows.

The apparent pH values for a series of four or five buffers (pH 6–7 or pH 7–8) were determined with the capillary electrode using the same standard pH 7·00. This standardization and series of pH determinations were then repeated seven times so that eight values of pH were obtained for each solution. The mean of the eight readings (C) is compared in Table 1 with the corresponding reading from the Hartree electrode (H). From the data calibration curves were constructed to enable the true pH to be obtained from the values given by the capillary electrode.

Table 1. *Performances of three capillary electrodes* (C) *in the* pH *range* 6–8

(The pH values (H) of a series of phosphate buffers were determined with a Hartree (1952) glass electrode using a standard phosphate buffer (pH 7·00). The same buffers were then examined with the capillary electrode (C) using the same pH 7·00 standard. The mean values for eight determinations are given together with the standard errors.)

Electrode no. 0 (345 MΩ)			Electrode no. 2			Electrode no. Ax (240 MΩ)		
pH (H)	pH (C)	S.E.	pH (H)	pH (C)	S.E.	pH (H)	pH (C)	S.E.
6·18	6·24	0·028	—	—	—	—	—	—
6·39	6·43	0·023	—	—	—	—	—	—
6·60	6·62	0·017	—	—	—	—	—	—
6·83	6·82	0·018	—	—	—	—	—	—
7·21	7·18	0·007	7·20	7·21	0·011	7·20	7·17	0·023
7·42	7·39	0·047	7·40	7·40	0·009	7·42	7·36	0·021
7·61	7·51	0·008	7·61	7·56	0·023	7·61	7·52	0·020
7·82	7·70	0·014	7·80	7·80	0·017	7·82	7·70	0·009
—	—	—	—	—	—	8·01	7·85	0·030

For determining the pH of small quantities of body fluids, such as blood from the stomach of mosquitoes, the capillary containers had the advantage over other methods, not only that the volume required was so small, but also that the fluid was collected in the container in which the determination was made, which obviates loss through transference. An additional advantage in determining the pH of blood over the drop methods such as those described by Païc (1938), Chinoy (1947) or Dietz (1948), is that the surface area exposed to the air is small, so that gaseous diffusion is considerably reduced.

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ABSENCE OF POTENTIATION BETWEEN QUININE AND PYRIMETHAMINE IN INFECTIONS OF *PLASMODIUM GALLINACEUM* IN CHICKS

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I. INTRODUCTION

In 1953, Ray, Misra, Chandrasekhar & Singh described the occurrence of a high degree of potentiation between pyrimethamine and quinine when tested upon infections of *Plasmodium gallinaceum* in young chicks. When given concurrently, the dose of each drug could be reduced to $\frac{1}{64}$th of the minimum effective dose (M.E.D.) without reduction of antimalarial activity, though smaller doses of both drugs were ineffective. A dose of pyrimethamine $\frac{1}{128}$th the M.E.D. was, however, effective when given with $\frac{1}{4}$th the M.E.D. of quinine. A less marked synergistic action between these drugs was described in the treatment of thirty cases of *P. falciparum* malaria infection (Singh, Ray, Misra & Nair, 1953).

In view of the therapeutic interest of these results the combined action of the drugs has been studied upon the normal strain of *P. gallinaceum* maintained in the Molteno Institute.

II. METHODS

A series of chicks 4–10 days old, 45–60 g. in weight, was inoculated intravenously with 50 million parasites per bird. The drugs were given orally by catheter tube, the first dose or doses being given immediately after inoculation, and the remaining doses morning and evening on each of the following 3 days. Quinine was given as an aqueous solution of the bihydrochloride. Pyrimethamine was dissolved in water with the addition of a little lactic acid. The doses were calculated in mg./20 g. of body weight of bird. The different doses of each of the drugs, or of both the drugs, were tested upon groups of five birds, one group in each series receiving no drug and serving as a control. The activity of the drugs was assessed by a comparison of the proportion of infected erythrocytes per 500 in blood films made from the drugged and control birds on the morning of the fourth day following inoculation.

III. EXPERIMENTS

The maximum dose of quinine tested in these experiments was 0·6 mg.; this dose had a pronounced effect upon parasitaemia and corresponds closely to the M.E.D. (0·64 mg./20 g.) recorded by Ray and his co-workers. The maximum dose of pyrimethamine tested was 0·001 mg. which suppressed parasitaemia almost completely; this was a slightly higher dose than the M.E.D. (0·0006 mg.) as assessed by Ray and his colleagues, which was the highest dose tested by them.

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In the first experiment (Table 1), 0·6 mg. quinine and 0·001 mg. pyrimethamine, given in combined doses, had a completely suppressive action. This was not unexpected, as either dose alone had a marked suppressive effect. Although in the first experiment a combined dose of 0·4 mg. quinine and 0·0005 mg. pyrimethamine had a strongly suppressive action, in the second experiment its effect was less marked, and in both experiments either drug alone in these doses had a slight suppressive effect. The effect of smaller combined doses was approximately the same as that of similar doses of pyrimethamine alone.

Table 1. *The effect of quinine and pyrimethamine, alone or in combined doses, upon infections of* Plasmodium gallinaceum *in chicks*

(Q = quinine; P = pyrimethamine. Doses are expressed in mg. per 20 g. body weight. The intensity of infection is expressed as the average number of infected erythrocytes in 500 in groups of five birds.)

	Expt. 1	Expt. 2
Control	265	436
Quinine: 0·6	22	22
0·4	103	261
0·2	223	353
Pyrimethamine: 0·001	2	3
0·0005	78	130
0·0001	201	397
0·00005	—	373
Q + P: 0·6 + 0·001	0	—
Q + P: 0·4 + 0·0005	3	50
Q + P: 0·2 + 0·0001	181	390
Q + P: 0·1 + 0·00005	—	331

IV. DISCUSSION

Greenberg (1949) found that the action of proguanil upon *P. gallinaceum* was potentiated by sulphadiazine and other *p*-aminobenzoic acid (*p*A.B.) competitors, provided that they possessed antimalarial action, but not by quinine, pamaquin, chloroquine or mepacrine. Proguanil and sulphadiazine are believed to be closely related in their mode of antimalarial action, since sulphadiazine-resistant strains of *P. gallinaceum* proved to be highly resistant to proguanil (Bishop & McConnachie, 1950), an observation confirmed in *P. berghei* by Rollo (Rollo, 1951) and Thurston (1953). No cross-resistance to quinine was shown by these strains. The mode of action of proguanil and sulphadiazine, though related, is not identical, since whereas a sulphadiazine-treated strain of *P. gallinaceum* or *P. berghei* readily became resistant to proguanil, on one occasion only has resistance to sulphadiazine been observed in a proguanil-resistant strain of *P. gallinaceum*; also whereas the action of sulphadiazine upon *P. gallinaceum* is antagonized competitively by *p*A.B., attempts to antagonize the action of proguanil upon this parasite by this compound have either failed (Bishop, 1954) or only partially succeeded (Greenberg, 1953).

The mode of action of pyrimethamine appears to resemble that of proguanil closely, since resistance between these compounds appears to be reciprocal,

though resistance to both drugs may not develop with equal rapidity or intensity. Thus cross-resistance to pyrimethamine was observed in proguanil-resistant strains of *P. gallinaceum* by Robertson, Davey & Fairley (1952) and Rollo (1955), and in *P. knowlesi* by Singh, Ray, Basu & Nair (1952). Cross-resistance to proguanil has been observed in pyrimethamine-resistant strains of *P. gallinaceum* and *P. berghei* by Rollo (quoted by Thurston, 1953), in *P. berghei* by Thurston (1953) and in *P. cynomolgi* by Schmidt & Genther (1953). A strain of *P. knowlesi*, highly resistant to pyrimethamine, was also resistant to proguanil but not to quinine or sulphadiazine (Singh, Nair & Ray, 1954). The action of pyrimethamine (Rollo, 1955) and proguanil (Greenberg, 1953; Bishop, 1954) upon *P. gallinaceum* was inhibited by folic acid, though less effectively than that of sulphadiazine. Pyrimethamine, like proguanil, potentiates the antimalarial action of sulphadiazine, though no potentiation was observed between pyrimethamine and proguanil (Rollo, 1955).

The conclusion to be drawn from these results is that pyrimethamine, proguanil and sulphadiazine are closely related in their mode of action which is assumed to be directed against the *p*-A.B.-folic-folinic acid metabolic chain. Potentiation probably occurs, as Rollo (1955) has recently pointed out, when two drugs act at different points on the same metabolic pathway, whereas an additive effect occurs when they act at the same point or on different pathways. The evidence from cross-resistance tests suggests that sulphadiazine acts at a different point on the same pathway from proguanil and pyrimethamine. There is no evidence from cross-resistance tests that the antimalarial action of these compounds and quinine is related, and potentiation between pyrimethamine and quinine has not been observed between these drugs when tested in combined doses on the Molteno Institute strain of *P. gallinaceum*.

Results of tests for synergism between drugs which show a relationship in their mode of action may vary. Thus synergism between quinine and pamaquin was described by Reilly, Chen & Geiling (1950) in *P. lophurae*, but this was not confirmed in *P. gallinaceum*, although a four- to eightfold increase in resistance to pamaquin was obtained in a substrain of the strain on which the combined action of the drugs was studied, and the pamaquin-resistant strain showed some cross-resistance to quinine (Bishop & McConnachie, 1952).

V. SUMMARY

The effect of combined doses of pyrimethamine and quinine has been studied in *P. gallinaceum*. No potentiation of the action of the drugs was observed.

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GRAHAMELLA (RICKETTSIALES) IN THE COMMON SHREW *SOREX ARANEUS*

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(With 1 Figure in the Text)

INTRODUCTION

Brumpt (1911) defined the genus and species *Grahamella talpae*, but he attributed the discovery of this blood parasite to Graham-Smith (1905), who described it in the erythrocytes of some moles caught near Cambridge. Within the next few years, Franca (1911) found *Grahamella* in *Talpa europaea* and *T. caeca* of Portugal, and Coles (1914), Laveran & Marullaz (1914) and Visentini (1913) found it in moles of England, France and Italy respectively. Yakimoff (1929) and Kikuth (1932) both stated that Wrublewsky (1912) found it in moles too, but this paper describes an organism which is quite evidently not *Grahamella*. *Grahamella* has also been recorded in other Insectivora: in the common shrew *Sorex vulgaris* (=*S. araneus*) of France by Laveran & Marullaz (1914), in the water shrew *Crossopus* (*Neomys*) *fodiens* of England by Henry (1913), in the shrew *Crocidura stampflii* of Senegal by Léger (1917), and in the short-tailed shrew *Blarina brevicauda* of U.S.A. by Tyzzer (1942). There are scattered records of *Grahamella* in non-mammalian hosts: Yakimoff (1929) described it in the perch *Perca fluviatilis* [of ?], Cerruti (1930) in the tortoise *Testudo graeca* and (1931) in the tortoise *Talassochelys* [*Thalassochelys*] *caretta* of Sardinia, and Carpano (1935) in a Leghorn cock of Egypt. There are also records of *Grahamella* in the bat *Desmodus rufus* of Peru by Ribeyro & del Aguila (1918), in the bat *Hemiderma brevicauda* of Brazil by Faria & Pinto (1926) [who wrongly identified the parasite as *Bartonella*], in a bull *Bos taurus* of Russia by Marzinowsky (1917), and in a wolf *Canis lupus* [of Russia ?] by Kamalov (1933). There is a single record of *Grahamella* in a Primate: it was found in *Macacus rhesus* of Indo-China by Léger (1922).

In contrast, there are more than seventy records of *Grahamella* in various rodents. Sixty-five of these are listed in Table 1, using the classification adopted by Ellerman (1940–9). Eight other records by Russian authors were listed by Yakimoff (1929), but are not included in the table because I have been unable to see them myself, and because in any case some of the information reported by Yakimoff is inadequate or contradictory.

The following are notes on the records of Table 1:

Cricetulus sp. [?] by Dudtschenko (1914), who gave only a vague description of the animal. This identity was suggested in an abstract by Mesnil (1915), and by Yakimoff (1929).

Cricetus phoca by Yakimoff (1917). I have been unable to identify this animal, nor does Ellerman list *phoca* as a specific name in any rodent genus.

Table 1. *Records of* Grahamella *in Rodents*

Host's name (Ellerman)	Host's name (Author)	Author	Locality
Family Sciuridae			
Citellus pygmaeus	*Citellus pygmaeus*	Sassuchin (1931*b*)	South Russia
Family Heteromyidae			
Perognathus californicus	*Perognathus californicus ochrus*	Wood (1952)	U.S.A.
Family Dipodidae			
Alactaga major	*Alactaga jaculus*	Sassuchin (1931*a*)	Astrakhan
Alactaga sibirica	*Alactaga mongolica*	Jettmar (1932)	Manchuria, Mongolia
Dipus sagitta	*Dipus sowerbyi*	Jettmar (1932)	Manchuria, Mongolia
Jaculus sp.	Jerboa	Balfour (1906)	Sudan
Jaculus orientalis	*Jaculus orientalis*	Laveran & Marullaz (1914)	Tunisia
Family Muscardinidae			
Eliomys quercinus	*Eliomys quercinus*	Franca (1911)	Portugal
Eliomys quercinus	*Myoxus nitela*	Laveran & Marullaz (1914)	France
Eliomys quercinus	*Myoxus nitela*	Bruynoghe & Vassiliadis (1930)	France
Family Muridae			
Micromys minutus	*Mus minutus*	Bruynoghe & Vassiliadis (1929, 1930)	France
Apodemus sp.	*Sylvaemus* sp.	Sassuchin (1931*a*)	Saratov, Russia
Apodemus sp. [?]	Fieldmouse	Kikuth (1932)	[?]
Apodemus sylvaticus	*Mus sylvaticus*	Laveran & Marullaz (1914)	France
Apodemus sylvaticus	Fieldmouse	Coles (1914)	England
Apodemus sylvaticus	*Mus sylvaticus*	Bruynoghe & Vassiliadis (1929, 1930)	France
Apodemus agrarius	*Apodemus agrarius*	Jettmar (1932)	Manchuria, Mongolia
Pelomys fallax	*Golunda fallax*	Joyeux (1913)	French Guinea
Rattus rattus	*Mus rattus*	Joyeux (1913)	French Guinea
Rattus rattus	*Epimys rattus*	Macfie (1917)	Gold Coast
Rattus norvegicus	*Mus maurus*	Léger (1913)	Upper Senegal
Rattus norvegicus [?]	Rat	Coles (1914)	England
Rattus norvegicus [?]	Brown rat	Macfie (1914)	Nigeria
Rattus norvegicus	*Mus decumanus*	Macfie (1915)	Gold Coast
Rattus norvegicus	*Mus decumanus*	Carini (1915)	Brazil
Rattus norvegicus [?]	Brown rat	Macfie (1916)	Gold Coast
Rattus norvegicus	*Rattus norvegicus (decumanus)*	Nauck (1927)	China
Rattus norvegicus	*Mus norvegicus*	Vassiliadis (1934)	Egypt
Rattus hawaiiensis	*Rattus hawaiiensis*	Kartman (1954)	Hawaii
Rattus coucha	*Mastomys coucha*	Neitz (1938)	South Africa
Mus musculus	*Mus musculus* var. *albinos*	Benoit-Bazille (1920)	France
Mus musculus	*Mus musculus wagneri*	Sassuchin (1931*a*)	Western Kazakhstan

Table 1 (*continued*)

Host's name (Ellerman)	Host's name (Author)	Author	Locality
Family MURIDAE (*continued*)			
Acomys cahirinus	*Mus acomys*	Vassiliadis (1934)	Egypt
Cricetomys gambianus	*Cricetomys gambianus*	Rodhain (1915)	Belgian-Congo
Cricetomys gambianus	Pouched rat	Macfie (1916)	Gold Coast
Cricetomys gambianus	Pouched rat (*Cricetomys gambianus*)	Macfie (1917)	Gold Coast
Peromyscus maniculatus	*Peromyscus maniculatus gracilis*	Tyzzer (1942)	U.S.A.
Peromyscus leucopus	*Peromyscus leucopus novaboracensis* [*novaeboracensis*]	Tyzzer (1942)	U.S.A.
Akodon serrensis	*Acodon* [*sic*] *serrensis*	Carini (1924)	Brazil
Cricetulus sp. [?]	?	Dudtschenko (1914)	Ural Steppes
Cricetulus barabensis	*Cricetulus griseus*	Patton & Hindle (1926)	China
Cricetulus barabensis	*Cricetulus furunculus* *Cricetulus griseus fumatus*	Jettmar (1932)	Manchuria, Mongolia
Cricetulus triton	*Cricetulus triton*	Jettmar (1932)	Manchuria, Mongolia
Phodopus roborovskii	*Phodopus bedfordiae*	Jettmar (1932)	Manchuria, Mongolia
Cricetus cricetus	*Cricetus cricetus*	Sassuchin (1931*a*)	Uralsk
[?]	*Cricetus phoca*	Yakimoff (1917)	Transcaucasia
Phyllotis darwinii	*Phyllotis darwini* [*sic*] *linatus* [*limatus*]	Tyzzer (1942)	from Peru
Meriones tristrami	*Meriones tristrami*	Adler (1930)	Palestine
Meriones tamaricinus	*Gerbillus tamaricinus*	Sassuchin (1931*a*)	Western Kazakhstan
Microtus pennsylvanicus	*Microtus pennsylvanicus*	Tyzzer & Weinman (1939)	U.S.A.
Microtus pennsylvanicus	*Microtus pennsylvanicus pennsylvanicus*	Tyzzer (1942)	U.S.A.
Microtus arvalis	*Microtus arvalis*	Lavier (1921)	France
Microtus arvalis	*Arvicola arvalis*	Bruynoghe & Vassiliadis (1929, 1930)	France
Microtus agrestis [?]	*Microtus incertus*	Franca (1911)	Portugal
Microtus agrestis	*Microtus agrestis*	Henry (1913)	England
Microtus agrestis	Field vole	Coles (1914)	England
Microtus gregalis *Lasiopodomys brandti*	*Microtus raddei* *Microtus brandti*	[?] Jettmar (1932)	Manchuria, Mongolia
Pitymys pinetorum	*Pitymys pinetorum*	Tyzzer (1942)	U.S.A.
Arvicola amphibius	Water vole	Coles (1914)	England
?	*N. bandicota*	O'Brien (1912)	Gold Coast
?	Yellow mouse	Prowazek (1913)	West Africa
?	Rat	Connall & Coghill (1916)	Nigeria
?	Field rat	Macfie (1917)	Gold Coast
?	Vole	Yakimoff (1917)	Transcaucasia

Microtus incertus of Portugal by Franca (1911). The geographical distribution of this species is said by Miller (1912) to be 'Mountains of Switzerland and Tirol, from central Alps eastward'. *M. arvalis* is similar in appearance and more widespread, but the western limit of its range is said to be the Pyrenees. It is therefore probable that Franca was dealing with *M. agrestis*, the next nearest relative.

M. raddei and *M. brandti* by Jettmar (1932), who gave the former name in the summary but used the latter in the text. Both species have suitable distributions.

N. bandicota by O'Brien (1912). By '*N*' he might have meant *Nesokia*; but *Nesokia bandicota* is a rat of India not Africa.

The details given by the various authors of all these records vary considerably. Some merely noted the occurrence of *Grahamella* in the blood of a particular host, but others gave some circumstantial details and bestowed on the parasite a specific name. (These names are omitted because the validity of so many is doubtful, for the reasons given later.) In contrast, some authors denied the parasitic nature of Graham-Smith's bodies, and preferred to regard them as basophil granules resulting from some sort of nuclear breakdown. Laveran & Marullaz (1914) were of this opinion, but their paper included a short description of these bodies in the erythrocytes of the shrew *Sorex vulgaris* (= *S. araneus*) of France. I have found no other record of *Grahamella* in *Sorex*.

OBSERVATIONS

Thirty-three specimens of the shrew *Sorex araneus* Linn. were trapped during August 1953 at Rosta in north Norway (68° 58′ N., 19° 42′ E.). Some of the specimens had died in the traps some hours before being examined. Each was weighed, measured and sexed; and a little of the blood from inside the heart was used to make a smear. The smears were stained with Leishmann's stain.

Nine of the shrews were found to be infected with *Grahamella*. The parasites had the morphological appearance of other members of this genus described in other species of small mammals. Infected erythrocytes contained organisms which were in the form of dots, wedges and dumb-bells. The most frequent forms were the dumb-bells (Fig. 1), which were typically about 1 μ long and about 0·2–0·3 μ broad. The stained organisms were unicoloured, but the swollen ends of the dumb-bells were stained a deeper purple than the middle parts. Similarly, the dots were a uniform deep purple, and the thick ends of the wedges were stained more deeply than the thin. Infected erythrocytes contained from three to twenty-six organisms but there were rarely fewer than ten, and the average number for all nine infected animals was seventeen. The proportion of infected erythrocytes varied in the different shrews, ranging up to an estimated 4 %. The incidence did not appear to be related to the age or sex of the host.

Some voles trapped at the same place and time were similarly examined. This collection comprised nine specimens of *Clethrionomys rutilus* Pallas, nine *Microtus oeconomus ratticeps* Keyserling & Blasius and one *Microtus agrestis* Linn. None was found to be infected with *Grahamella*. No other parasites were distinguished in any of the blood smears examined.

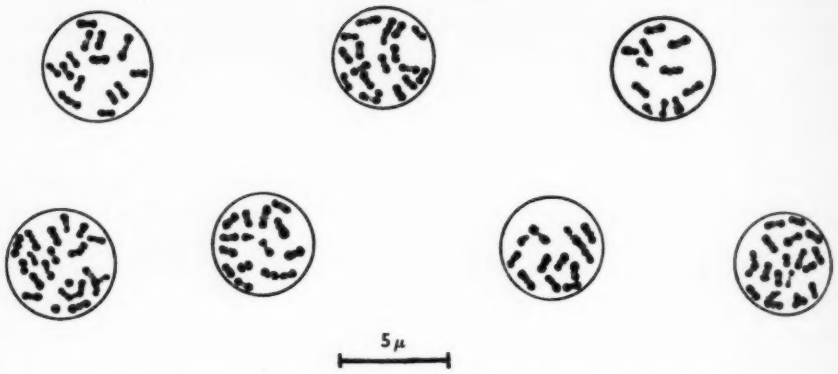

Fig. 1. Some erythrocytes of the shrew parasitized by *Grahamella*.

DISCUSSION

Tyzzer (1942) listed four characters used as criteria in the recognition of species of *Grahamella* and related erythrocytic parasites. They were (1) host of origin, (2) morphology, (3) pathogenicity, (4) cultural characteristics. The differentiation of species of *Grahamella* itself rests on the first and the last characters, since all members of this genus are morphologically rather alike, and are without any marked pathogenic effect on the host.

The majority of authors have offered no direct evidence of the infectivity for other hosts of the *Grahamella* they described. Negative evidence was given by Léger (1913) and Neitz (1938) who injected new hosts with infected blood, and by O'Brien (1912) who used this test and the more biological one of transferring the ecto-parasites from infected to non-infected hosts. These three authors and Adler (1930) failed to transmit *Grahamella* to hosts of the same species, but Jettmar (1932) succeeded, using cultures of *Grahamella*. Vassiliadis (1934) also succeeded, using direct inoculation of infected blood. Presumably in ignorance of Jettmar's work, he claimed the first success in thus unmistakably proving the parasitic nature of these bodies, and he also made the more extraordinary claim that his paper constituted the first record of *Grahamella* in rats. Vassiliadis does, however, appear to be the first author to have transmitted *Grahamella* from one species of host to another: from *Mus* (=*Rattus*) *norvegicus* to *Mus* (=*Rattus*) *rattus*. Later Tyzzer (1942) succeeded in transmitting *Grahamella* from one species of deer mouse to another and from a vole to a white mouse.

Negative evidence of an indirect sort is offered by various authors who examined several more or less taxonomically related animals from the same locality and failed to find *Grahamella* in all species. However, negative evidence of this sort is not very convincing since Bruynoghe & Vassiliadis (1930), Jettmar (1932) and Tyzzer (1942) showed that the parasite may disappear from the peripheral blood either permanently or in a cyclical fashion. Thus a single examination of a host may easily miss an infection which is permanently or temporarily hidden. So

the figures given by many authors on the proportion of a population infected with the parasite are probably of little value. This permanent hiding can be considered as an acquired immunity, and Tyzzer (1942) suggested that an experimental failure to infect an 'uninfected' host may be based on such immunity. Brumpt (1949) also put forward this explanation, and the alternative one that the infection may become hidden immediately in the new host and never be apparent to the experimenter.

Because of these considerations, and because of the widespread occurrence of *Grahamella* amongst rodents, it is probable that amongst this group at least several species of hosts may harbour the same species of parasite. It is probably better to regard the multiplicity of species of *Grahamella* (one per species of host) with some misgiving and to hold that most of these specific names are of doubtful validity.

The strong morphological resemblance between *Grahamella* of different host-origins is another reason for caution. On the other hand, it must be admitted that the simple structure of the parasite does not in any case give much scope for morphological variation; and its environment for much of its life-cycle is the interior of the erythrocyte, itself morphologically much alike in the different hosts. However, an interesting variation in the descriptions of *Grahamella* in various hosts is that only Graham-Smith (1905), Ribeyro & del Aguila (1918), Faria & Pinto (1926) and Patton & Hindle (1926) seem to have found chromatin granules in the parasites. This may be accident due to differences in stains and techniques used by different authors. In any case Brumpt (1911), when defining the new genus, was careful to state that it has the characters described in his paper and in Graham-Smith's.

The shapes of the parasites are reported by most authors to be rounded or bacilliform. Usually both forms are present, but some reports mention only the latter type. The ends of the bacilliform bodies are usually heavily stained, giving the appearance of dumb-bells rather than rods, whereas the spherical and oval bodies are uniformly heavily stained. Brumpt (1911) suggested that the parasite reproduces by transverse division, and that the bacillary bodies are division forms. He remarked that he had seen very few of the irregular forms reported by Graham-Smith (1905). The latter had stated that: 'The rods are never straight, and many have a marked curve in them, often near one of the extremities. One or both ends of the longer forms are enlarged, giving the organisms a wedge- or club-shaped appearance. Some of the medium-sized forms are definitely dumb-bell shaped, the very small ones almost round.' Nevertheless, his amazingly detailed illustrations do show a number of rods which are straight. Irregular forms were also seen by Coles (1914) and Patton & Hindle (1926). Faria & Pinto (1926) saw irregular forms of a parasite they named *Bartonella rocha-limai*. Since the organisms occupied the interior of the erythrocytes they could not have been true *Bartonella*; and the description strongly suggests that these authors saw a *Grahamella*. Bruynoghe & Vassiliadis (1929) reported that they sometimes encountered a ring-form, looking like a sporulating *Bacillus subtilis*, and suggested that it might be a development phase. Dudtschenko (1914) found rodlets and parallelograms. He

named the parasite *Theileria parva*, but again the description strongly suggests that a *Grahamella* was seen. On the other hand, the parasite named *G. gallinarum* by Carpano (1935), who found it in a Leghorn cock, cannot have been a *Grahamella*. Its morphology was extremely varied, but the chief objection is that it was Gram positive. Cerruti (1930, 1931) described two species of *Grahamella*, in *Testudo graeca* and *Talassochelys* [*sic*] *caretta*, but it is very doubtful if either belongs in the genus. Although both had forms of varied shapes, no rods or dumb-bells were observed. The bodies in *Testudo* were certainly of the right order of size, but those which were in the other tortoise and which were said to be similar in appearance were much larger (rings 3–6 μ in diameter; oval, curved and reniform bodies 2·5–5·5 μ long). The bodies in *Testudo* occupied 25 % of the erythrocytes, whereas all other authors are agreed in finding parasitized corpuscles to be rare. (It is difficult to assess the percentage of parasitized corpuscles in their various preparations since most were content to state simply that they found one per so many fields, without necessarily stating the magnification of eye-piece or objective.) The bodies in both tortoises were motile, whereas Laveran & Marullaz (1914) stated, as a result of their investigations, that Graham-Smith's bodies were neither motile nor even visible in fresh preparations. Cerruti observed, at most, a mixed infection of *Grahamella* and some other parasite.

These three wrong identifications are all of parasites occurring in non-mammalian hosts. A fourth identification, which is not quite so easy to discard, is that of Yakimoff's (1929). He found *Grahamella* in a perch which, he stated, was 'vom See "Pidmozero"', but I am unable to find the whereabouts of this lake. He found up to only twelve parasites per erythrocyte, whereas other authors have found considerably more, the erythrocyte sometimes being crammed with them. There is nothing to rule out the possibility that the 'parasitized' corpuscles were in fact merely degenerate. He stated that their outlines were irregular and that their nuclei were small and pale. Apart from this, it is unlikely that *Grahamella* should occur in a host which is taxonomically so remote from all others.

Bartonella ranarum of the frog *Leptodactylus ocellatus*, described by Cunha & Muniz (1927), was classified by Kikuth (1932) as a *Grahamella*. It is true that, occurring within the erythrocytes, the parasite cannot be a *Bartonella*, but not enough information is given to warrant including it in the genus *Grahamella*. In any case, the rods were stated to be up to 2 μ long, but the majority of authors have described rods of 1 μ or less in length, and there are few records of rods as large even as 1·5 μ. The only exception is the 2–2·5 μ long rods of *Grahamella* in the bat *Desmodus rufus* described by Ribeyro & del Aguila (1918). *Bartonella ranarum* must therefore remain of uncertain classification, as must all these other bodies in non-mammalian hosts which can be excluded for various reasons from the genus *Grahamella*. The corollary of this is that members of the genus *Grahamella* are known to parasitize mammalian hosts only.

The only record I can find of *Grahamella* being found in an arthropod is that of Marzinowsky (1917) who, the abstract states, found it in the intestines of examples of the tick *Margaropus* (*Boophilus*) *calcaratus* taken from a parasitized bull. Graham-Smith (1905) found no ticks but a number of fleas, lice and mites

on the moles he examined. There is no direct evidence as to whether *Grahamella* has an arthropod vector.

The pathogenic effect of *Grahamella* on its mammalian hosts is thought by most authors to be negligible or non-existent. However, Dudtschenko (1914) remarked that infected animals amongst the ones he examined often died of fright in the traps. O'Brien (1912) noted that an infected '*N. bandicota*' died in the sixth week of captivity, having shown signs of drooping since the third. He suggested that the cause of death was a sudden and general liberation of parasites from the erythrocytes. However, post-mortem examination revealed only a few infected erythrocytes in the heart-blood and bone-marrow. Graham-Smith (1905) examined the bodies of his infected moles and found a more general distribution of infected corpuscles: in the liver, lung, spleen and kidney, and a very few in the bone-marrow. More evidence is provided by Benoit-Bazille (1920), Lavier (1921), and Carini (1924) who noted that parasitized erythrocytes are to some extent dehaemoglobinized. On the other hand, Léger (1913), Macfie (1914) and Sassuchin (1931*a*, *b*) all stated that the staining properties of parasitized erythrocytes were not affected. The majority of authors have failed to note any enlargement of parasitized erythrocytes, but Graham-Smith (1905) said that those containing more than twenty organisms were enlarged, and Léger (1922) noted that about one in ten were slightly hypertrophied. Coles (1914) thought that infected erythrocytes first enlarged and then burst, and Jettmar (1932) was able to follow the whole of this cycle of development. Parasitized erythrocytes are destroyed, but since the proportion attacked is low there may be no noticeable effect on the host. Indeed, as noted above, it may suppress the parasite. This may conceivably be reversed by a major stress on the host, but most authors are agreed that even splenectomy is without effect. Tyzzer (1942), who had considerable experience, found that this operation increased the numbers of *Grahamella* only in one deer mouse he examined. A more marked effect was noted by Nauck (1927), who found that splenectomy revealed *Grahamella* in three out of six *Rattus norvegicus*, and by Neitz (1938), who used splenectomy to reveal *Grahamella* in all six of the *Mastomys* (= *Rattus*) *coucha* he examined.

If there is a pathogenic effect on the host of any significance, it might be expected to vary with the season or the age of the host. Information on this interesting point is extremely scanty. Kartman (1954) failed to find any such relation, but he suggested that sex had some influence on infection rate since the twenty-seven *Rattus hawaiiensis* that he found to be infected were in the proportion of nine males to eighteen females. The infections in the *Sorex araneus*, described earlier in this paper, seemed to be unrelated to the age or the sex of the hosts.

Attempts to culture *Grahamella* were for a long time unsuccessful and gave some authors grounds for doubting its parasitic nature. Macfie (1917) made a Bass culture and met with limited success, but Jettmar (1932) and Tyzzer (1942), also using blood-rich media, both developed good cultures. The latter author remarked that '...too few animals have been injected with cultured grahamella from other species to establish host specificity as a wholly reliable basis for

differentiating species of grahamella. The distinctive characters observed in grahamella of various host species when propagated in culture media offer more conclusive evidence as to the multiplicity of grahamella species.'

SUMMARY

Nine out of thirty-three specimens of the common shrew *Sorex araneus*, caught at Rosta in north Norway in August 1953, were found to be infected with *Grahamella* (Rickettsiales). Nine specimens of *Clethrionomys rutilus*, nine of *Microtus oeconomus ratticeps* and one of *M. agrestis*, caught at the same time and place, were found to be free of *Grahamella*. No other blood parasites were found in any of the animals examined.

Records by other authors of *Grahamella* in a variety of hosts are reviewed. The parasite is known to occur in mammalian hosts only: identifications of it in non-mammalian hosts can be discarded. Host specificity, tacitly assumed by most authors, is not proven, and therefore most of the specific names bestowed on *Grahamella* in different hosts are of doubtful validity. The morphology of the parasite varies little from host to host; and there is little evidence to show that its pathogenic effect on any host is serious. *Grahamella* has been cultured by a few authors: variations in characters of these cultures may be more reliable criteria for recognition of different species of *Grahamella*.

These small mammals were collected while I was a member of the University of Edinburgh Expedition to Arctic Norway, 1953. I wish to thank all who were concerned, in particular Dr D. M. Steven, with whom I did the trapping. The rest of the work was done during the tenure of a Research Studentship awarded by the Agricultural Research Council. I also wish to thank Dr P. Tate for his advice and his criticisms of the manuscript.

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SOME VIRUSES AFFECTING INSECTS OF ECONOMIC IMPORTANCE

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(With Plates III–VI)

INTRODUCTION

The success attending some attempts to control certain insect pests by the artificial propagation of virus diseases, notably the alfalfa caterpillar in California (Thompson & Steinhaus, 1950), and the pine sawfly in Canada (Bird, 1953), stimulated our search for viruses affecting other important insect pests, both in this country and abroad.

Many of the viruses, briefly described here, are newly recorded, and it is surprising how soon a virus affecting a particular insect can be found if a sufficiently careful search for it is made. An exception to this was the difficulty experienced over 4 years in finding viruses which would attack the larvae of the two white butterflies, *Pieris brassicae* and *P. rapae*. However, in the summer of 1955 a granulosis virus was found infecting the larvae of both species, which is among the most virulent we have seen. At the moment we are not able to state definitely whether one or two granulosis viruses are concerned in the diseases of the two species.

The year 1955 was marked by a very heavy invasion of white butterflies, mostly *P. brassicae*, from the continent, and we suspect that among these immigrants were some which carried the virus with them and transmitted it through the egg to their offspring.

CROSS-INFECTION EXPERIMENTS AND LATENT VIRUSES

In a previous paper the results were given of cross-transmission experiments with a virus from *Vanessa cardui* to some thirty miscellaneous species of Lepidoptera (Smith & Xeros, 1954*a*). In some cases, the inoculated larvae developed a cytoplasmic polyhedral disease in response to inoculations with a nuclear virus. This could be interpreted in one of two ways: either there was present in the inoculum a small amount of cytoplasmic virus, or a latent cytoplasmic virus was stimulated to active development by the introduction of a foreign virus.

In view of these results cross-infection experiments were carried out with certain insect pests which had not developed a virus disease of their own, in the hope of obtaining successful cross-transmission. One such case was the winter moth, *Operophtera brumata*, for which no natural virus infection had been found. During 1954 some hundreds of larvae of this species were fed on foliage heavily contaminated with nuclear polyhedra from *Vanessa cardui*. After a period of 10–20 days the larvae began to die of a virus disease, the final mortality being

nearly 100%. Examination under the microscope revealed the presence of a cytoplasmic polyhedrosis (Pl. IV, fig. 6). All the control larvae remained healthy. Similarly, with the larvae of *Bupalus piniarius*, the pine looper; these were given the same virus with the same result. The following year (1955) the experiments with both species were repeated, but this time with almost negative results. All the winter-moth larvae remained healthy and only a small percentage of the pine loopers died of a cytoplasmic virus infection. During 1955, however, a number of dead pine looper larvae were sent to the laboratory from the Forest Commission at Alice Holt Lodge, and examination of these revealed the presence of a cytoplasmic polyhedrosis (Pl. IV, fig. 7) indistinguishable from the cytoplasmic disease of the previous year, apparently resulting from infection with a virus from *Vanessa cardui*.

One interpretation of these results could be that the 1954 stocks of larvae of *Operophtera brumata* and *Bupalus piniarius* were almost completely infected with a latent cytoplasmic polyhedrosis which was stimulated or 'triggered' into action by the introduction of a foreign virus. The nearly negative results of the 1955 inoculations would, on this theory, be interpreted to mean that the larvae were not carrying a latent cytoplasmic virus. The fact that *B. piniarius* larvae were found in 1955 dying of a cytoplasmic virus shows that a natural infection of this type does exist, and thus supports the theory of a stimulated latency rather than a genuine cross-infection.

Some preliminary cross-transmission experiments with the granuloses of the larvae of the two white butterflies are of interest in this connexion. Of 100 larvae of *Pieris brassicae* fed on foliage contaminated with the granulosis virus from *P. rapae*, forty-three died of a granulosis, whilst of the 100 control larvae seventeen died of granulosis over a period of 5 days. It is, perhaps, significant that in a parallel experiment in which *P. brassicae* were infected with their own virus, the mortality was 100%, and the larvae commenced to die in 72 hr.

The inference here is that the *rapae* granulosis virus is 'triggering' the latent *brassicae* virus into action. The question, however, as to whether there are two viruses involved or only one transmissible between the two species will have to wait until fresh stocks of the larvae of both species are available.

In the large number of cross-transmission experiments carried out, by far the greater number of polyhedral diseases developing in the inoculated insects were the cytoplasmic infections of the gut. It is suggested, therefore, that latent viruses are more often of the cytoplasmic rather than the nuclear type.

DESCRIPTION OF SOME OF THE VIRUS DISEASES

The viruses found attacking insects fall into four groups: (1) nuclear polyhedral viruses; (2) cytoplasmic polyhedral viruses; (3) granuloses or capsular disease viruses; (4) viruses without intracellular inclusions. All four types have been isolated from the insects under study, and altogether twenty-six viruses are recorded. Most of these are either new or only recently discovered.

It is not practicable to give detailed descriptions of all these viruses and the

diseases they cause; one or two diseases and their causative viruses have therefore been selected for more comprehensive treatment, whilst a few relevant details are given about the remaining diseases.

The granulosis viruses

In diseases caused by this type of virus the characteristic inclusion consists of an outer capsule or granule, within which is usually another capsule containing the rod-shaped virus particle. In some granuloses there may be more than one virus particle in the inner capsule. The virus particles appear to be always rod-shaped. In these studies six granulosis viruses are included. One of them, affecting the so-called nettle grub of tea (*Natada nararia*) has already been briefly described (Smith & Xeros, 1954*b*). The other five were isolated from the caterpillars of the following insects: *Euplexia leucipara*, the small angleshades; *Agrotis segetum* (Schiff.), the cutworm or turnip moth; *Melanchra persicariae*, the dot moth; *Pieris brassicae* (L.), the large white butterfly and *P. rapae*, the small white butterfly. A detailed description is given only of the last three.

Pieris brassicae L. (the large white butterfly)

The granulosis virus which we have isolated from infected larvae of *P. brassicae* is in all probability the same as that originally described by Paillot (1926). He observed the granules in the cytoplasm only of the cells of the fat-body and hypodermis.

Under laboratory conditions the onset of the disease is very rapid, and young larvae begin to die 72 hr. after infection. Older larvae may take a little longer. The first indication of infection is loss of appetite; infected larvae immediately stop feeding and remain quiescent. A pallor next develops, which is most marked in the thoracic region, and death rapidly ensues after this. The larva becomes flaccid and hangs down in a characteristic manner, rather in the shape of an inverted V (Pl. IV, fig. 5). The skin is extremely fragile and ruptures at a touch, liberating the contents of the body which have become almost entirely liquefied. It is a curious fact that these liquefied corpses seem to have a strong attraction for other uninfected caterpillars which feed readily upon them, with, as may be imagined, disastrous consequences.

In occasional cases of death from this disease the body of the caterpillar does not rupture at a touch and the skin appears normal. In such cases it seems that the hypodermis is not affected and the granules may be confined to the fat-body.

Larvae in various stages of the disease have been examined by means of sections fixed in Carnoy's solution and stained with iron haematoxylin. The stages were 72 and 96 hr. after infection, and older larvae which were in a late stage of the disease showing the characteristic pallor. Studies of sections of caterpillars at these different stages of infection seem to support Paillot's suggestion that the granules occur in the hypodermis (Pl. III, fig. 2) and fat-body. Furthermore, it seems as if the virus develops in the nucleus rather than in the cytoplasm. In sections through the fat-body of a young larva of *P. brassicae*, 96 hr. after infection, the nuclei appear to be full of granules and there are indications that they

rupture, liberating a mass of granules into the cytoplasm of the cell. This seems to be similar to the development of the granulosis virus in the buckeye caterpillar, *Junonia coenia* Hübner (Steinhaus & Thompson, 1949).

Pl. III, fig. 2, is part of a section through the hypodermis of a larva of *P. brassicae* in an advanced stage of the disease. The whole background is a mass of granules.

The granules themselves are shown in Pl. V, fig. 9, at a magnification of 15,300 times, after treatment with 1 % sodium carbonate. The outer capsule has collapsed, revealing the second inner capsule which contains the rod-shaped virus particle.

Pieris rapae L. (the small white butterfly)

The progress of the granulosis disease in this species is apparently very similar to that in the large white butterfly. In Pl. III, fig. 1, is seen a section of fat-body from an infected larva of *P. rapae*; it shows the enormously enlarged nucleus which is presumably the centre of virus development. Sections through the hypodermis show hypertrophy of the cells and the presence of many granules.

Pl. V, fig. 12, is an electron micrograph of the granules, treated with 1 % sodium carbonate. They are similar in appearance to the granules from *P. brassicae*. What is probably the same virus has been recorded by Thompson (1951) in California.

Melanchra persicariae L. (the dot moth)

In this species, also, the disease seems to follow a similar course to that in the two white butterflies. The site of multiplication of the virus is almost certainly nuclear. Pl. III, figs. 3 and 4, are sections through the fat-body of a caterpillar of *M. persicariae* in a fairly advanced stage of infection. The granules inside the greatly enlarged nucleus are clearly seen, and from one nucleus (Pl. III, fig. 4) the granules appear to be spilling out in large quantities into the cytoplasm.

The hypodermis is similarly affected and the cells become filled with granules.

VIRUSES IN THE CONTROL OF INSECT PESTS

Several of the insects listed in this paper for which viruses have been found are of considerable agricultural or economic importance. For example, the cutworm, the leatherjacket and the winter moth are notorious pests, whilst the damage done by the larvae of the two clothes moths is, of course, well known.

As a method of control the use of viruses has several advantages over the more orthodox chemical insecticide, provided certain difficulties can be overcome. The advantages are, first, the selective nature of the virus which kills only the insect in question and leaves unharmed the beneficial parasites or predators. Secondly, the polyhedral viruses are more persistent than any chemical and will remain infective under some conditions for periods of years. Thirdly, they are in a sense self-propagating since a disease once started will continue to spread, especially in crowded insect populations. In the case of the white butterfly we suspect that the granulosis virus is transmitted to subsequent generations and this, of course, would greatly increase the efficiency of control.

One of the difficulties in the use of viruses as a control measure lies in the production of large quantities. This is particularly true in the case of the clothes moth where the larva is comparatively small. A number of attempts has been made, without success, to propagate the virus in a larger species of lepidopterous larva. One interesting result of these inoculation experiments was the appearance of a virus disease in some of the species inoculated. This virus, however, could not be returned to the clothes-moth larvae, thus suggesting the stimulation or 'triggering' into action of a latent virus by the presence of a foreign one.

Another difficulty is inherent in the habitat of the insect, for example, the cutworm and the leatherjacket are soil-inhabiting larvae and it would not be easy to reach them with a virus.

The best chance of success may be expected in those cases where the larvae feed openly on the leaves of the crop, as with the caterpillars of the two species of white butterflies. Here, conditions favour the successful use of virus control. The caterpillar is a fairly large one and the amount of virus available from each larva is considerable. Furthermore, it has been shown by the Unit of Insect Physiology at Cambridge, whose co-operation in this matter we gratefully acknowledge, that the caterpillars of the large white butterfly, *Pieris brassicae*, will breed continuously throughout the winter under conditions of artificial lighting and heating. It is thus possible to produce and store up large quantities of virus during the winter for use in the following season.

Most of the damage by the caterpillars of the white butterflies is done by the second generation later in the year. Spraying with virus for control should therefore be done early in the season in order to start the disease going and so prevent the development of the second generation. The caterpillars feed openly, and often crowded together, on the leaves of brassica crops so that application of the virus by spraying should be easy. It may be necessary to add a 'sticker' to make the virus adhere to the rather waxy leaves of the food plant.

It is hoped also that some control may be effected, in a similar way, of the wattle bagworm in South Africa and the American cotton bollworm in Uganda. In these two cases nuclear polyhedral viruses are being used, and the work is being directed by the entomologist on the spot.

LIST OF ECONOMIC INSECTS AND THE VIRUSES FOUND AFFECTING THEM

LEPIDOPTERA

CARADRINIDAE		
(1) *Phlogophora meticulosa* (L.)	Angleshades moth	Cytoplasmic polyhedrosis
(2) *Euplexia lucipara* (L.)	Small angleshades moth	Granulosis (Pl. V, fig. 11)
AGROTINAE		
(3) *Agrotis segetum* Schiff.	Turnip moth or cutworm	Cytoplasmic polyhedrosis and granulosis (Pl. V, fig. 10)
(4) *Triphaena pronuba* (L.)	Yellow underwing	Cytoplasmic polyhedrosis
(5) *Heliothis armigera* Hueb.	American cotton bollworm (Uganda)	Nuclear and cytoplasmic polyhedrosis (Pl. VI, fig. 15)

LIST OF ECONOMIC INSECTS AND THE VIRUSES FOUND AFFECTING THEM

	LEPIDOPTERA (*cont.*)	
MELANCHRINAE		
(6) *Diataraxia oleracea* (L.)	Tomato moth	Cytoplasmic polyhedrosis
(7) *Melanchra persicariae* (L.)	Dot moth	Granulosis (Pl. III, figs. 3, 4)
LYMANTRIDAE		
(8) *Euproctis chrysorrhea* (L.)	Brown tail moth	Cytoplasmic polyhedrosis
SELIDOSEMIDAE		
(9) *Biston betularia* (L.)	Pepper and salt moth	Cytoplasmic polyhedrosis
(10) *Abraxas grossulariata* (L.)	Currant moth	Nuclear and cytoplasmic polyhedrosis (Pl. IV, fig. 8)
(11) *Bupalus piniarius* (L.)	Pine looper	Cytoplasmic polyhedrosis (Pl. IV, fig. 7)
HYDRIOMENIDAE		
(12) *Operophtera brumata* (L.)	Winter moth	Cytoplasmic polyhedrosis (Pl. IV, fig. 6)
PIERIDAE		
(13) *Aporia crataegi* (L.)	Black veined white butterfly	Cytoplasmic polyhedrosis
(14) *Pieris brassicae* (L.)	Large white butterfly	Granulosis (Pl. V, fig. 9)
(15) *Pieris rapae* (L.)	Small white butterfly	Granulosis (Pl. V, fig. 12)
HETEROGENCIDAE		
(16) *Natada nararia*	'Nettle grub' of tea (Ceylon)	Granulosis
PSYCHIDAE		
(17) *Acanthopsyche junodi*	Wattle bagworm (South Africa)	Nuclear polyhedrosis (Pl. VI, figs. 13, 14)
TINAEIDAE		
(18) *Tineola bisselliella* (Hummel)	Clothes moth	Nuclear polyhedrosis
(19) *Tinaea pellionella* (L.)	Case-bearing clothes moth	Nuclear and cytoplasmic polyhedrosis
	HYMENOPTERA	
DIPRIONIDAE		
(20) *Neodiprion sertifer* (Fourcroy)	Pine sawfly	Nuclear polyhedrosis
TENTHREDINIDAE		
(21) *Nematus olfaciens*	Blackcurrant sawfly	Nuclear polyhedrosis
	DIPTERA	
TIPULIDAE		
(22) *Tipula paludosa* (Meig.)	Leatherjacket	Nuclear polyhedrosis and a virus without intracellular inclusions (Pl. VI, fig. 16)

SUMMARY

An account is given of some viruses affecting insects of economic importance. Twenty-six viruses are listed affecting twenty-two species of insects. Of these viruses eleven cause cytoplasmic, and eight cause nuclear, polyhedroses. Six are granulosis viruses and one virus has no intracellular inclusions. Most of these

viruses are new or recently recorded. The granulosis viruses, affecting *Pieris brassicae*, *P. rapae* and *Melanchra persicariae*, and the diseases they cause, are described in some detail.

We are indebted to Dr L. L. J. Ossowski of the Wattle Research Institute, University of Natal, and to Mr T. H. Coaker of the Cotton Research Station, Namulonge, Uganda, who sent us, respectively, diseased specimens of the wattle bagworm and the American cotton bollworm, from which the viruses were isolated. Miss S. Vernon-Smith took the electron micrographs, and Mr Simon Frey the photographs on the optical microscope.

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EXPLANATION OF PLATES, III–VI

(Electron micrographs by S. Vernon-Smith; all shadowed with palladium-gold. Photo-micrographs by S. Frey.)

Plate III

Fig. 1. Section through fat-body of a larva of *Pieris rapae*; infected with a granulosis virus: note the greatly enlarged nucleus. × 825.

Fig. 2. Section through the hypodermis of a larva of *P. brassicae*; infected with a granulosis virus: note the masses of granules. × 825.

Fig. 3. Section through the fat-body of a larva of *Melanchra persicariae*; infected with a granulosis virus: note the enlarged nucleus and many granules. × 825.

Fig. 4. Similar section to that shown in fig. 3; note the nucleus, on the right, apparently liberating granules into the cytoplasm. × 825.

Plate IV

Fig. 5. Larvae of *Pieris brassicae*; infected with a granulosis virus, showing the characteristic appearance when dead.

Fig. 6. Section through the gut of a larva of the winter moth, *Operophtera brumata*, infected with a cytoplasmic polyhedrosis. The larva was inoculated with a polyhedral virus from *Vanessa cardui*. × 1100.

Fig. 7. Smear from a larva of *Bupalus piniarius*, the pine looper: natural infection with a cytoplasmic polyhedrosis. × 1100.

Fig. 8. Section through the gut of a larva of *Abraxas grossulariata*, infected with a cytoplasmic polyhedrosis. The larva was inoculated with a polyhedral virus from *Antitype xanthomista*. × 1100.

PLATE V

Fig. 9. Granules from a larva of *Pieris brassicae*, showing the outer and inner capsules: treated with 1% sodium carbonate for 30 sec. × 15,300.

Fig. 10. Granules from a larva of *Agrotis segetum*, the cutworm, showing the outer and inner capsules: treated with 1% sodium carbonate for ½ min. × 23,700.

Fig. 11. Granules from a larva of *Euplexia leucipara*, the small angleshades moth, showing the outer and inner capsules: treated with 1% sodium carbonate for 1 min. × 26,700.

Fig. 12. Granules from a larva of *Pieris rapae*: in this case the outer granule seems unusually large and may contain more than one inner capsule and virus rods: treated with 1% sodium carbonate for ¼ min. × 26,700.

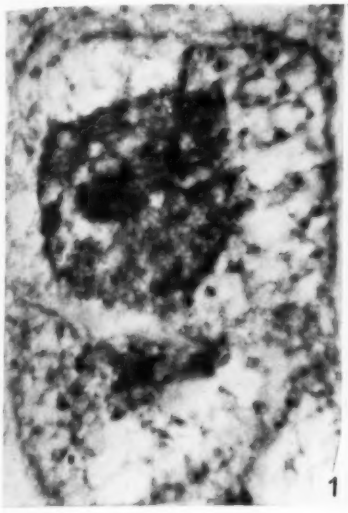
PLATE VI

Fig. 13. Polyhedral body from the nuclear polyhedrosis of the wattle bagworm, *Acanthopsyche junodi*, after treatment with 1% sodium carbonate for 5 min. Note the virus rods inside the residual membrane. × 28,800.

Fig. 14. Isolated virus particles from the same disease as in fig. 13: treated with 1% sodium carbonate for 2 min. Note the presence of some half-sized rods. × 28,800.

Fig. 15. Polyhedral body from the nuclear polyhedrosis of the American cotton bollworm, *Heliothis armigera* Hüb., after treatment with 1% sodium carbonate for 1 min. Some virus rods are visible inside the residual membrane and some, including half-size rods, are free. × 28,800.

Fig. 16. Virus particles from a larva of the crane fly, *Tipula paludosa* Meig., infected with a disease without intracellular inclusions. × 36,750.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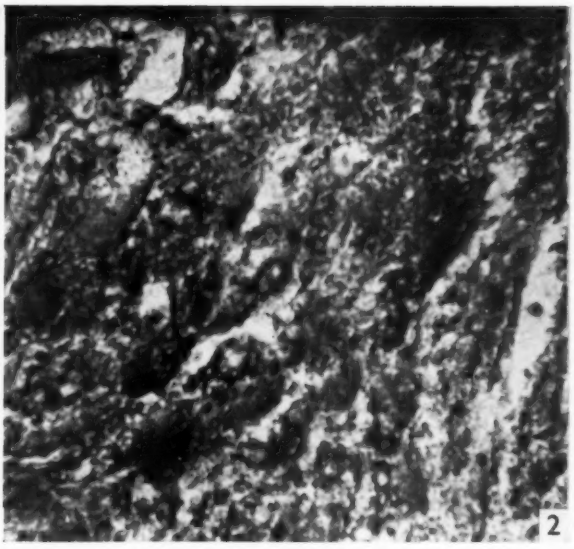
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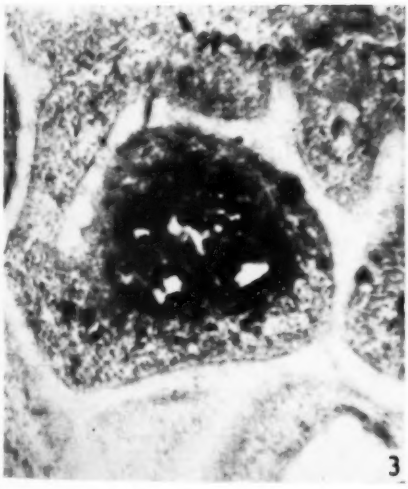
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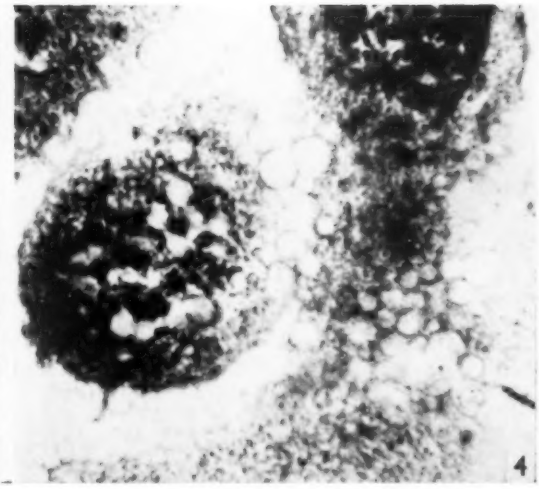
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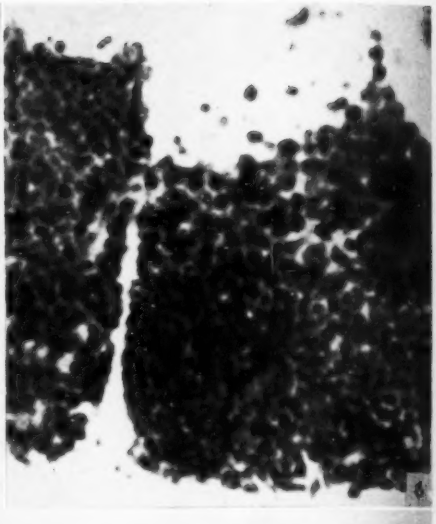
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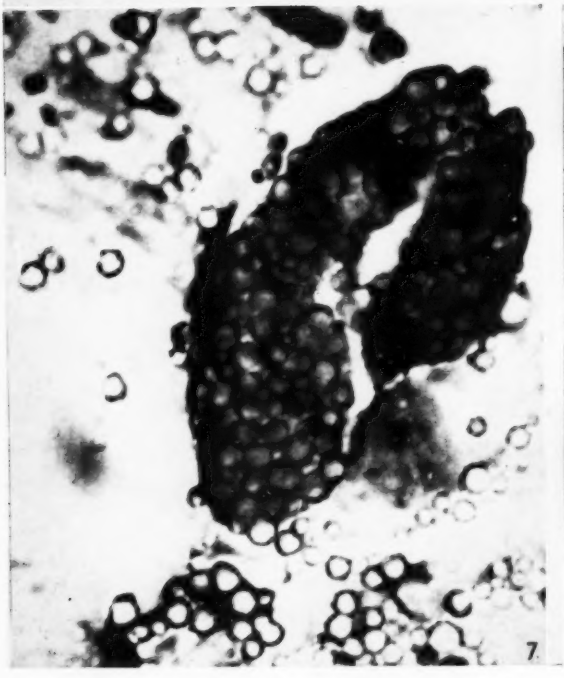
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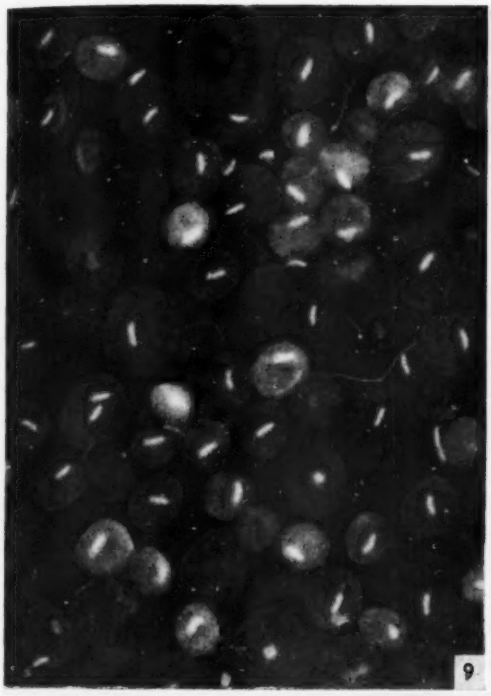
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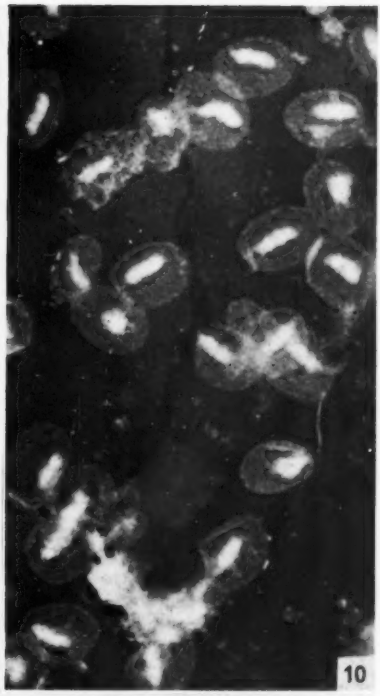
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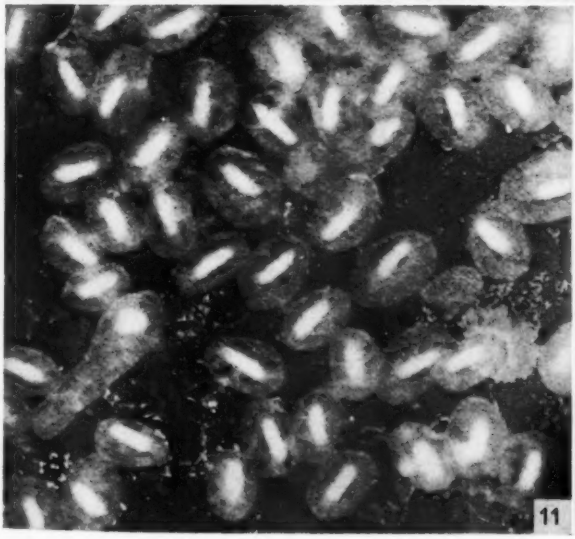
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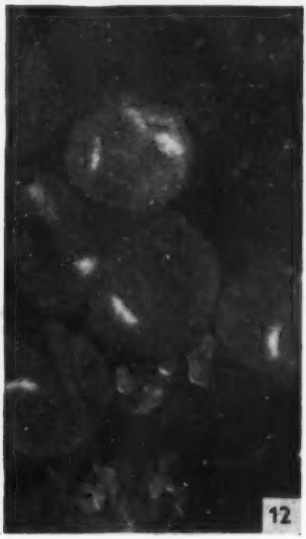
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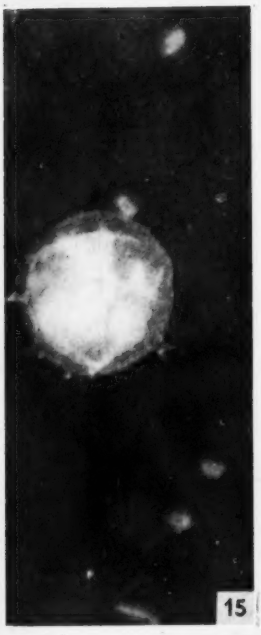
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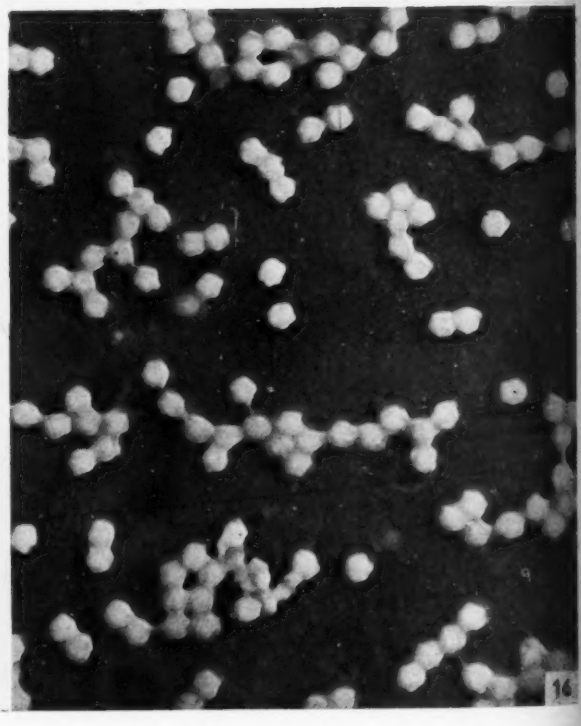
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